

## PATENT COOPERATION TREATY

From the  
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

FC  
SWABEY OGILVY RENAULT  
1981, Avenue McGill College  
Bureau 1600  
Montréal, Québec H3A 2Y3  
CANADA

PCT

NOTIFICATION OF TRANSMITTAL OF  
THE INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT  
(PCT Rule 71.1)

		Date of mailing (day/month/year) 13.12.2001
Applicant's or agent's file reference 1770-232PCT		<b>IMPORTANT NOTIFICATION</b>
International application No. PCT/CA00/01114	International filing date (day/month/year) 28/09/2000	Priority date (day/month/year) 30/09/1999
Applicant MCGILL UNIVERSITY		

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

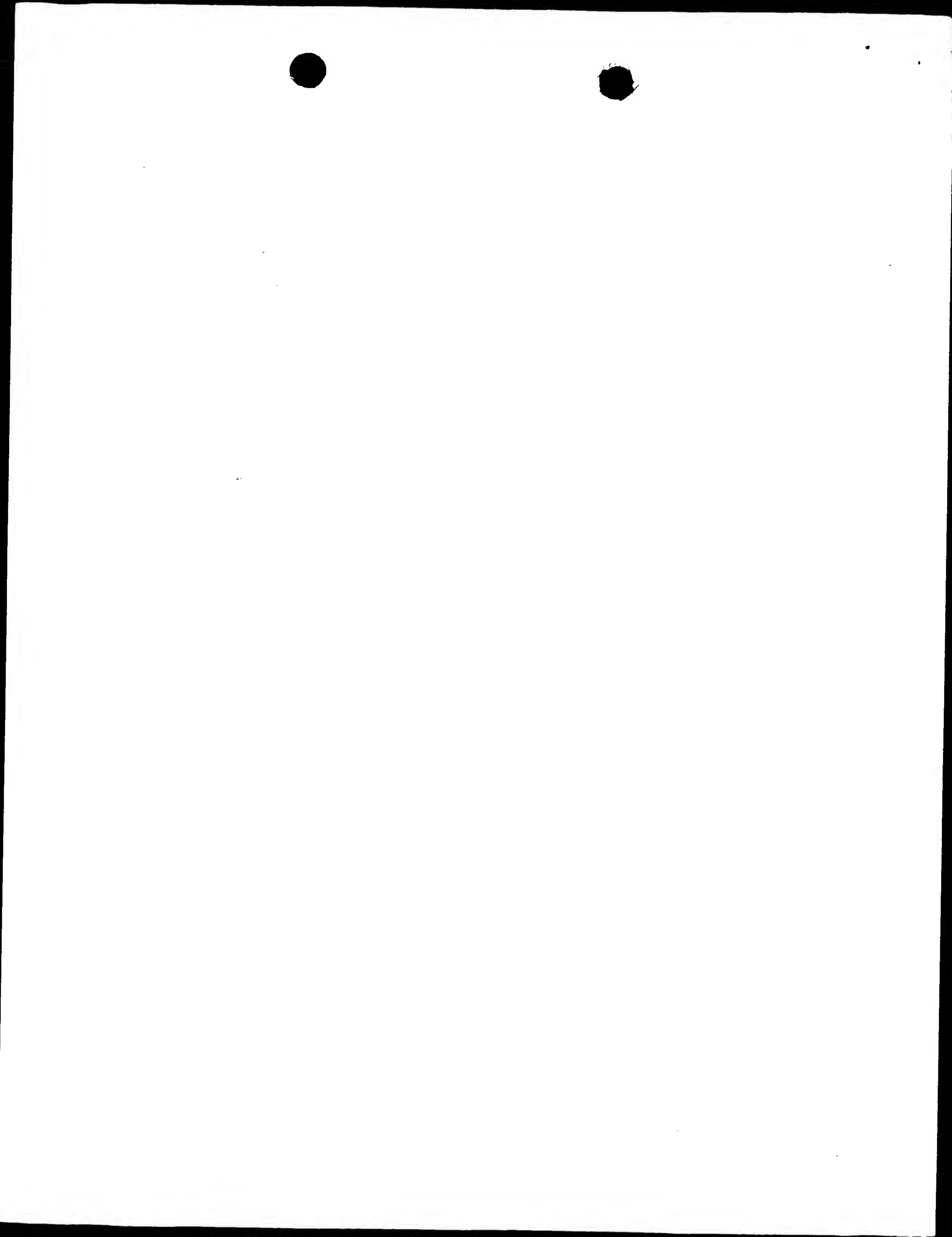
**4. REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/   European Patent Office D-80298 Munich Tel. +49 89 2390 - 0 Fax: 623056 epmu d Fax: +49 89 2399 - 4465	Authorized officer  Hundt, D  Tel.+49 89 2399-8042	
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## PATENT COOPERATION TREATY

PCT

## NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

Date of mailing (day/month/year) 05 April 2001 (05.04.01)	A.M. 7   8   9   10   11   12   1   2   3   4   5   6
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Applicant's or agent's file reference 1770-232PCT	IMPORTANT NOTICE
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International application No. PCT/CA00/01114	International filing date (day/month/year) 28 September 2000 (28.09.00)	Priority date (day/month/year) 30 September 1999 (30.09.99)
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Applicant MCGILL UNIVERSITY et al
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1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:  
AU,KP,KR,US

In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:  
AE,AG,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,BZ,CA,CH,CN,CR,CU,CZ,DE,DK,DM,DZ,EA,EE,EP,ES,  
FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,  
MN,MW,MX,MZ,NO,NZ,OA,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,  
The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the  
applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on  
05 April 2001 (05.04.01) under No. WO 01/22978

**REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)**

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, a demand for international preliminary examination must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

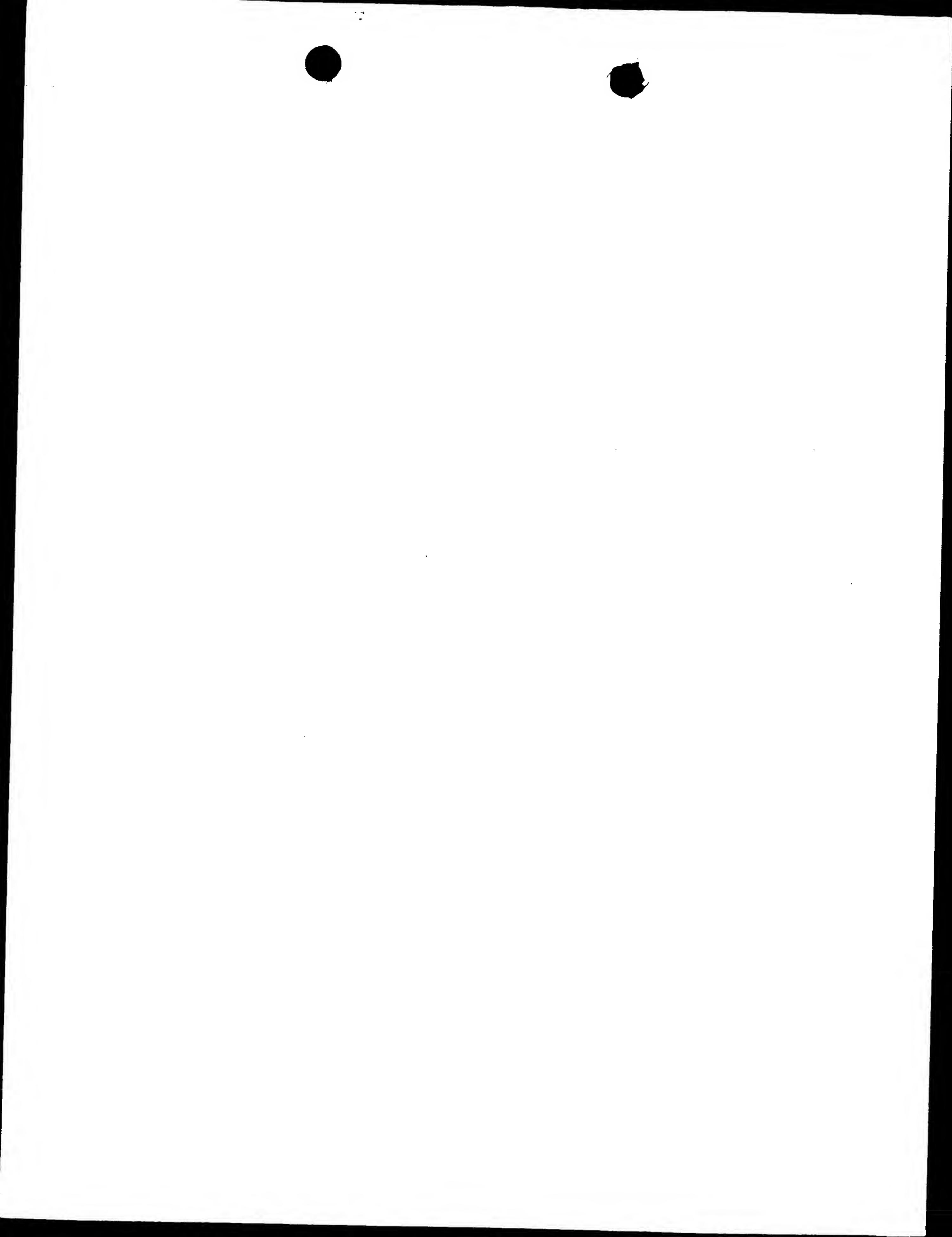
**REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))**

If the applicant wishes to proceed with the international application in the national phase, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland  Facsimile No. (41-22) 740.14.35	Authorized officer J. Zahra  Telephone No. (41-22) 338.83.38
--	---

3929285



## PATENT COOPERATION TREATY

US

From the INTERNATIONAL SEARCHING AUTHORITY

**PCT**

To:

SWABEY OGILVY RENAULT  
 1981, Avenue McGill College  
 Bureau 1600  
 Montréal, Québec H3A 2Y3  
 CANADA

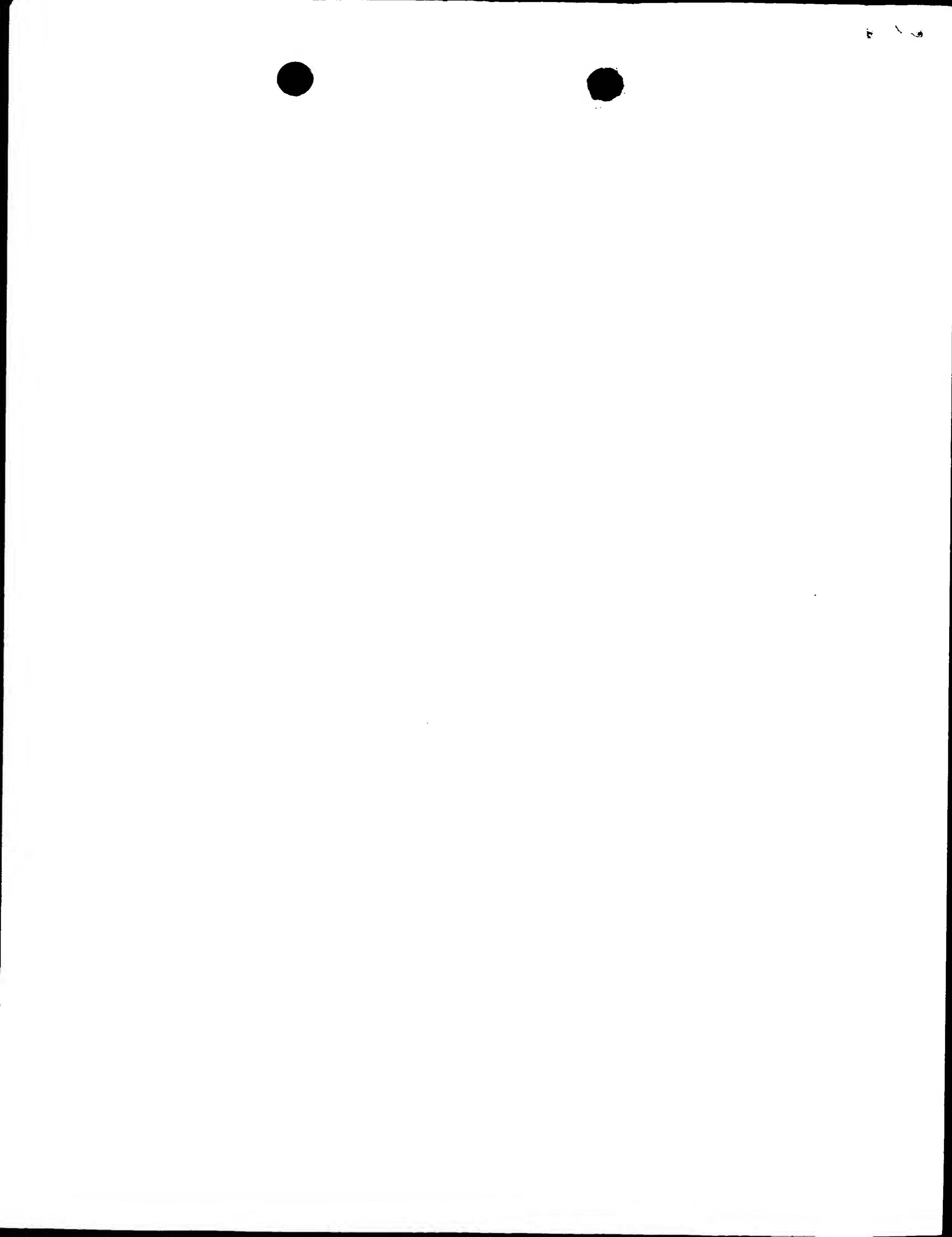
COMMUNICATION IN CASES FOR WHICH  
 NO OTHER FORM IS APPLICABLE

		Date of mailing (day/month/year)	18/05/2001
Applicant's or agent's file reference  1770-232PCT	<b>REPLY DUE</b>	See paragraph 1 below	
International application No.  PCT/CA 00/ 01114	International filing date (day/month/year)	28/09/2000	
<p>Applicant  MCGILL UNIVERSITY</p> <p>1. <input type="checkbox"/> REPLY DUE within _____ XXXX days from the above date of mailing  <input checked="" type="checkbox"/> NO REPLY DUE</p> <p>2. COMMUNICATION:       On 29.03.01 this International Searching Authority mailed the International Search Report for the above mentioned PCT-application to you. In this International Search Report a document has been cited incorrectly, namely WO 99 03937 A. This document is replaced by WO 99 03973 A.       Please find enclosed a new Search Report which cancels and replaces the one already in your possession, as well as copy of the document in question.       We want to apologize for any inconvenience caused.</p> <p>A copy of this letter and its enclosures has been sent to the International Bureau of W.I.P.O. in Geneva.</p>			

Name and mailing address of the International Searching Authority  
 European Patent Office, P.B. 5818 Patentlaan 2  
 NL-2280 HV Rijswijk  
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
 Fax: (+31-70) 340-3016

Authorized officer

Catherine Humbert



## PATENT COOPERATION TREATY

PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference <b>1770-232PCT</b>	<b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. <b>PCT/CA 00/ 01114</b>	International filing date (day/month/year) <b>28/09/2000</b>	(Earliest) Priority Date (day/month/year) <b>30/09/1999</b>
Applicant <b>MCGILL UNIVERSITY</b>		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

- a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
  - the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).
- b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :
  - contained in the international application in written form.
  - filed together with the international application in computer readable form.
  - furnished subsequently to this Authority in written form.
  - furnished subsequently to this Authority in computer readable form.
  - the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
  - the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2.  Certain claims were found unsearchable (See Box I).

3.  Unity of invention is lacking (see Box II).

4. With regard to the title,

- the text is approved as submitted by the applicant.
- the text has been established by this Authority to read as follows:

5. With regard to the abstract,

- the text is approved as submitted by the applicant.
- the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. \_\_\_\_\_

- as suggested by the applicant.
- because the applicant failed to suggest a figure.
- because this figure better characterizes the invention.

None of the figures.



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP A 00/01114

A. CLASSIFICATION OF SUBJECT MATTER  
 IPC 7 A61K35/28 A61P9/10

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
 IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 03937 A (DU PONT) 28 January 1999 (1999-01-28) the whole document ---	1-17
X	TOMITA S ET AL: "AUTOTRANSPLANTED MESENCHYMAL STEM CELLS IMPROVE FUNCTION AFTER A MYOCARDIAL INFARCTION" CIRCULATION, US, AMERICAN HEART ASSOCIATION, DALLAS, TX, vol. 98, no. SUPPL. 17, 1998, page ABS1036 XP000870314 ISSN: 0009-7322 the whole document ---	1-12 -/-



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

° Special categories of cited documents :

- 'A' document defining the general state of the art which is not considered to be of particular relevance
- 'E' earlier document but published on or after the international filing date
- 'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- 'O' document referring to an oral disclosure, use, exhibition or other means
- 'P' document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

15 March 2001

Date of mailing of the international search report

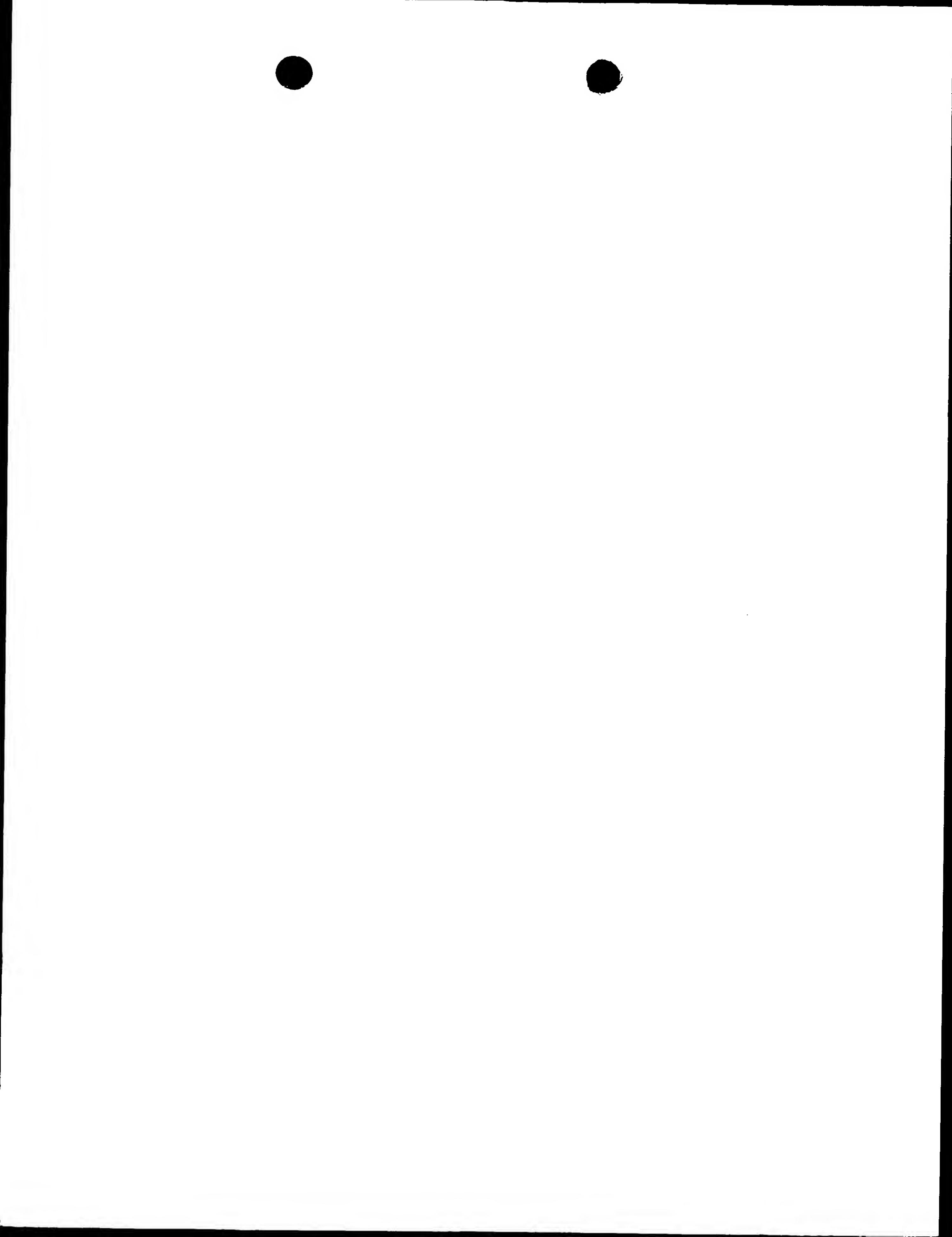
29/03/2001

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
 NL - 2280 HV Rijswijk  
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
 Fax: (+31-70) 340-3016

Authorized officer

Fernandez y Branas, F



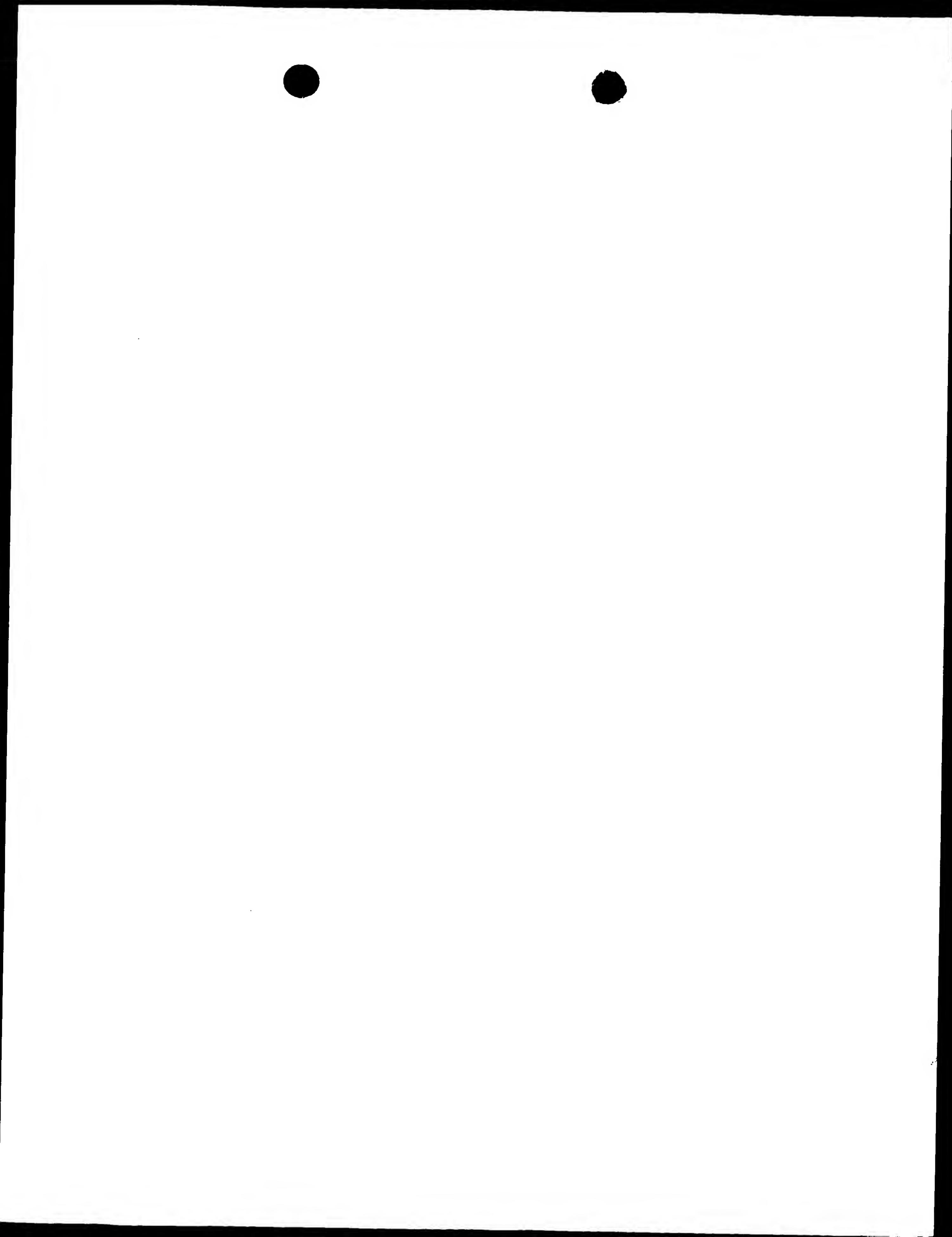
## INTERNATIONAL SEARCH REPORT

International Application No

PCT/A 00/01114

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MAKINO S. ET AL: "Cardiomyocytes can be generated from marrow stromal cells in vitro" THE JOURNAL OF CLINICAL INVESTIGATION, vol. 103, no. 5, March 1999 (1999-03), pages 697-705, XP000990317 the whole document ---	1-17
A	US 5 486 359 A (CAPLAN ARNOLD I ET AL) 23 January 1996 (1996-01-23) the whole document ---	1-17
A	PROCKPO D. ET AL: "Marrow stromal cells for nonhematopoietic tissues" SCIENCE, vol. 276, 1997, pages 71-74, XP002162972 the whole document ---	1-17
A	BRUDER S P ET AL: "MESENCHYMAL STEM CELLS IN BONE DEVELOPMENT, BONE REPAIR, AND SKELETAL REGENERATION THERAPY" JOURNAL OF CELL BIOLOGY, US, ROCKEFELLER UNIVERSITY PRESS, NEW YORK, US, vol. 56, no. 3, 1994, pages 283-294, XP000865959 ISSN: 0021-9525 the whole document ---	1-17
A	FERRARI G ET AL: "Muscle regeneration by bone marrow -derived myogenic progenitors 'see comments! 'published erratum appears in Science 1998 Aug 14;281(5379):923!'" SCIENCE, US, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, vol. 279, 6 March 1998 (1998-03-06), pages 1528-1530, XP002098163 ISSN: 0036-8075 the whole document ---	1-17
A	KESSLER P D ET AL: "MYOBLAST CELL GRAFTING INTO HEART MUSCLE: CELLULAR BIOLOGY AND POTENTIAL APPLICATIONS" ANNUAL REVIEW OF PHYSIOLOGY, US, ANNUAL REVIEWS, INC., PALO ALTO, CA, vol. 61, 1999, pages 219-242, XP000865708 ISSN: 0066-4278 the whole document ---	1-17
A	US 5 736 396 A (HAYNESWORTH STEPHEN E ET AL) 7 April 1998 (1998-04-07) the whole document -----	1-17



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/00/01114

A. CLASSIFICATION OF SUBJECT MATTER  
 IPC 7 A61K35/28 A61P9/10

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
 IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 03973 A (OSIRIS THERAPEUTICS INC ;GORDON STEPHEN L (US); MACKAY ALASTAIR MO) 28 January 1999 (1999-01-28) the whole document ---	1-17
X	TOMITA S ET AL: "AUTOTRANSPLANTED MESENCHYMAL STEM CELLS IMPROVE FUNCTION AFTER A MYOCARDIAL INFARCTION" CIRCULATION, US, AMERICAN HEART ASSOCIATION, DALLAS, TX, vol. 98, no. SUPPL. 17, 1998, page ABS1036 XP000870314 ISSN: 0009-7322 the whole document --- -/-	1-12

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

## ° Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

15 May 2001

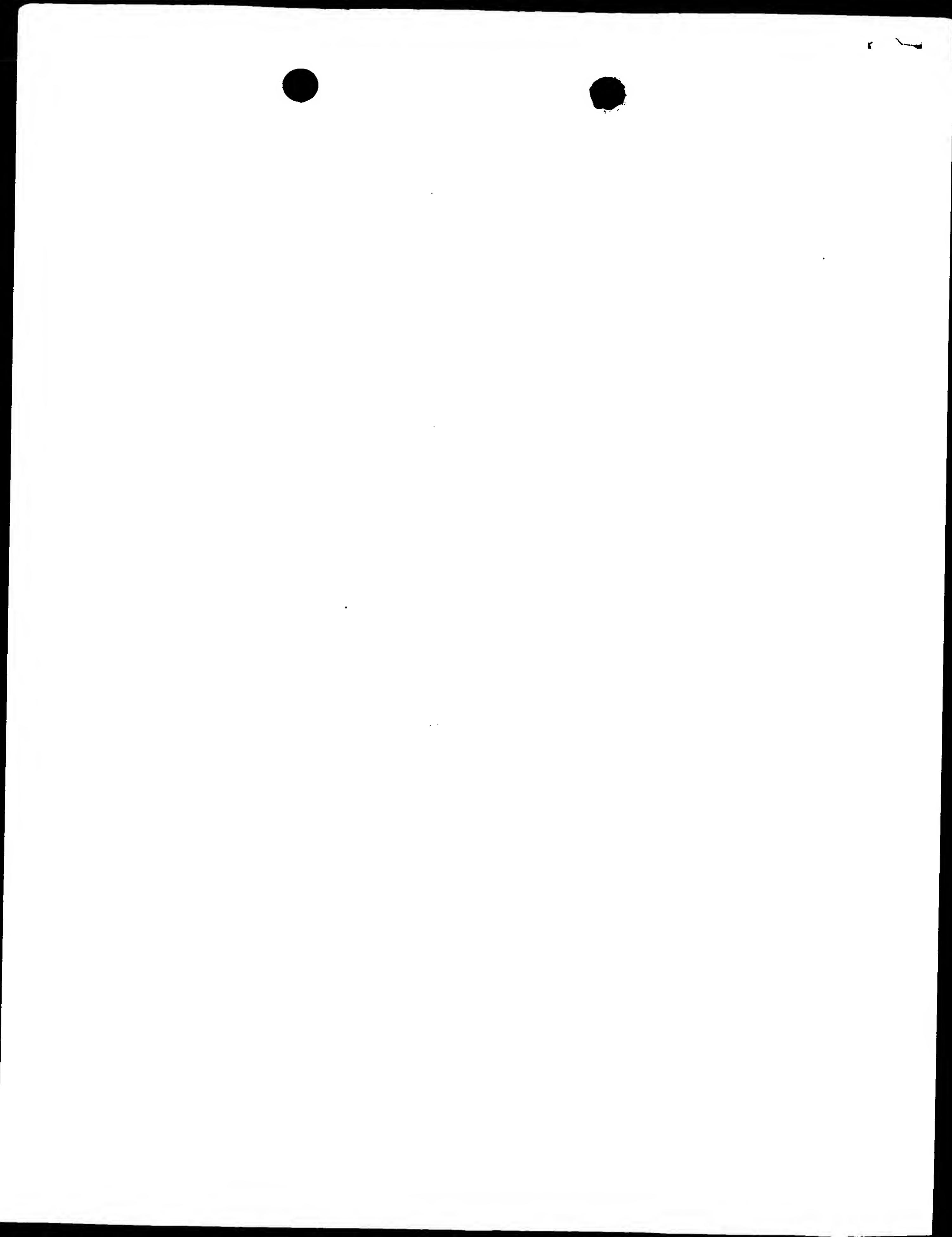
18.05.2001

## Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
 NL - 2280 HV Rijswijk  
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
 Fax: (+31-70) 340-3016

## Authorized officer

Fernandez y Branas, F



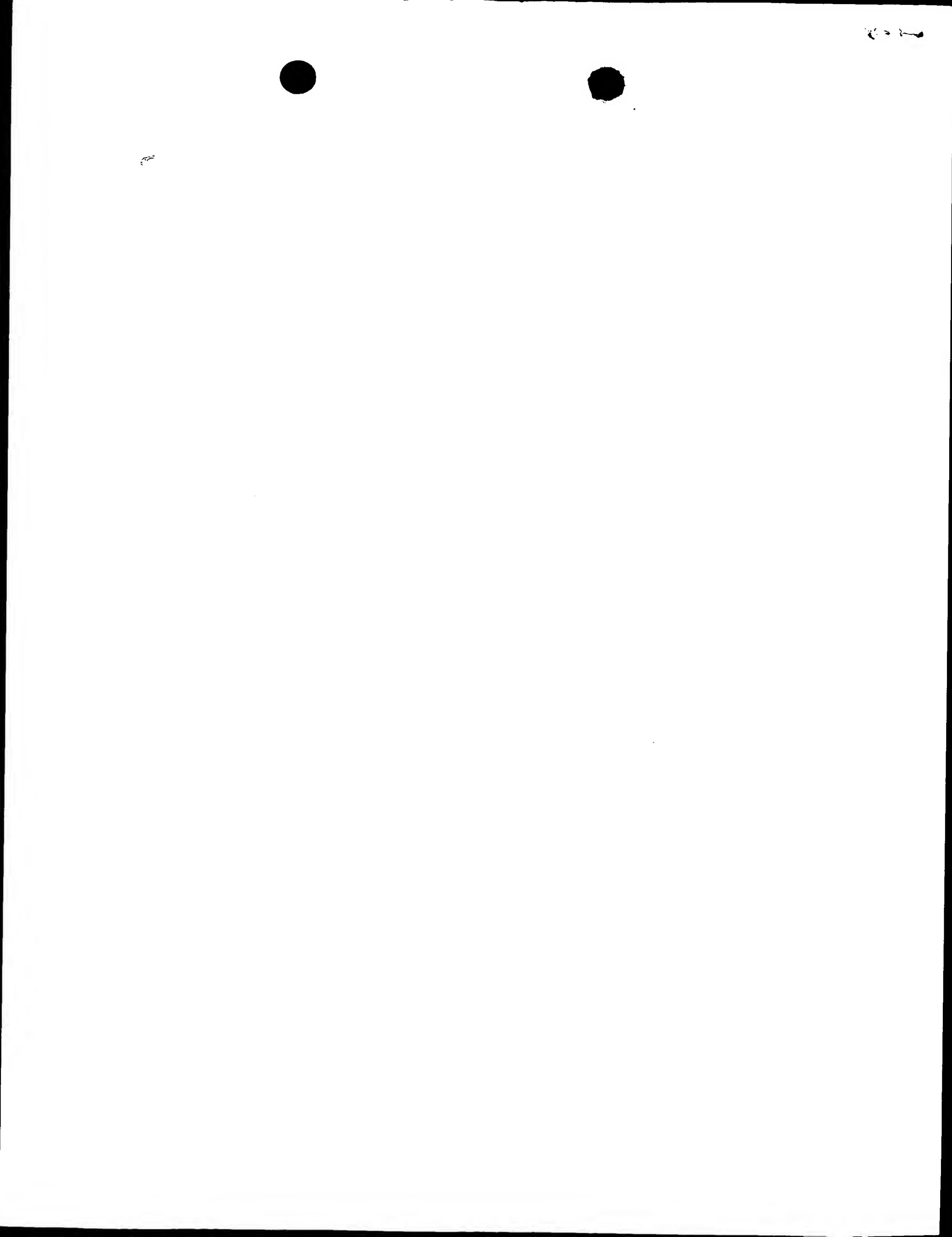
## INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 00/01114

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MAKINO S. ET AL: "Cardiomyocytes can be generated from marrow stromal cells in vitro" THE JOURNAL OF CLINICAL INVESTIGATION, vol. 103, no. 5, March 1999 (1999-03), pages 697-705, XP000990317 the whole document ---	1-17
A	US 5 486 359 A (CAPLAN ARNOLD I ET AL) 23 January 1996 (1996-01-23) the whole document ---	1-17
A	PROCKPO D. ET AL: "Marrow stromal cells for nonhematopoietic tissues" SCIENCE, vol. 276, 1997, pages 71-74, XP002162972 the whole document ---	1-17
A	BRUDER S P ET AL: "MESENCHYMAL STEM CELLS IN BONE DEVELOPMENT, BONE REPAIR, AND SKELETAL REGENERATION THERAPY" JOURNAL OF CELL BIOLOGY, US, ROCKEFELLER UNIVERSITY PRESS, NEW YORK, US, vol. 56, no. 3, 1994, pages 283-294, XP000865959 ISSN: 0021-9525 the whole document ---	1-17
A	FERRARI G ET AL: "Muscle regeneration by bone marrow -derived myogenic progenitors [see comments] [published erratum appears in Science 1998 Aug 14;281(5379):923]" SCIENCE, US, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, vol. 279, 6 March 1998 (1998-03-06), pages 1528-1530, XP002098163 ISSN: 0036-8075 the whole document ---	1-17
A	KESSLER P D ET AL: "MYOBLAST CELL GRAFTING INTO HEART MUSCLE: CELLULAR BIOLOGY AND POTENTIAL APPLICATIONS" ANNUAL REVIEW OF PHYSIOLOGY, US, ANNUAL REVIEWS, INC., PALO ALTO, CA, vol. 61, 1999, pages 219-242, XP000865708 ISSN: 0066-4278 the whole document ---	1-17
A	US 5 736 396 A (HAYNESWORTH STEPHEN E ET AL) 7 April 1998 (1998-04-07) the whole document -----	1-17



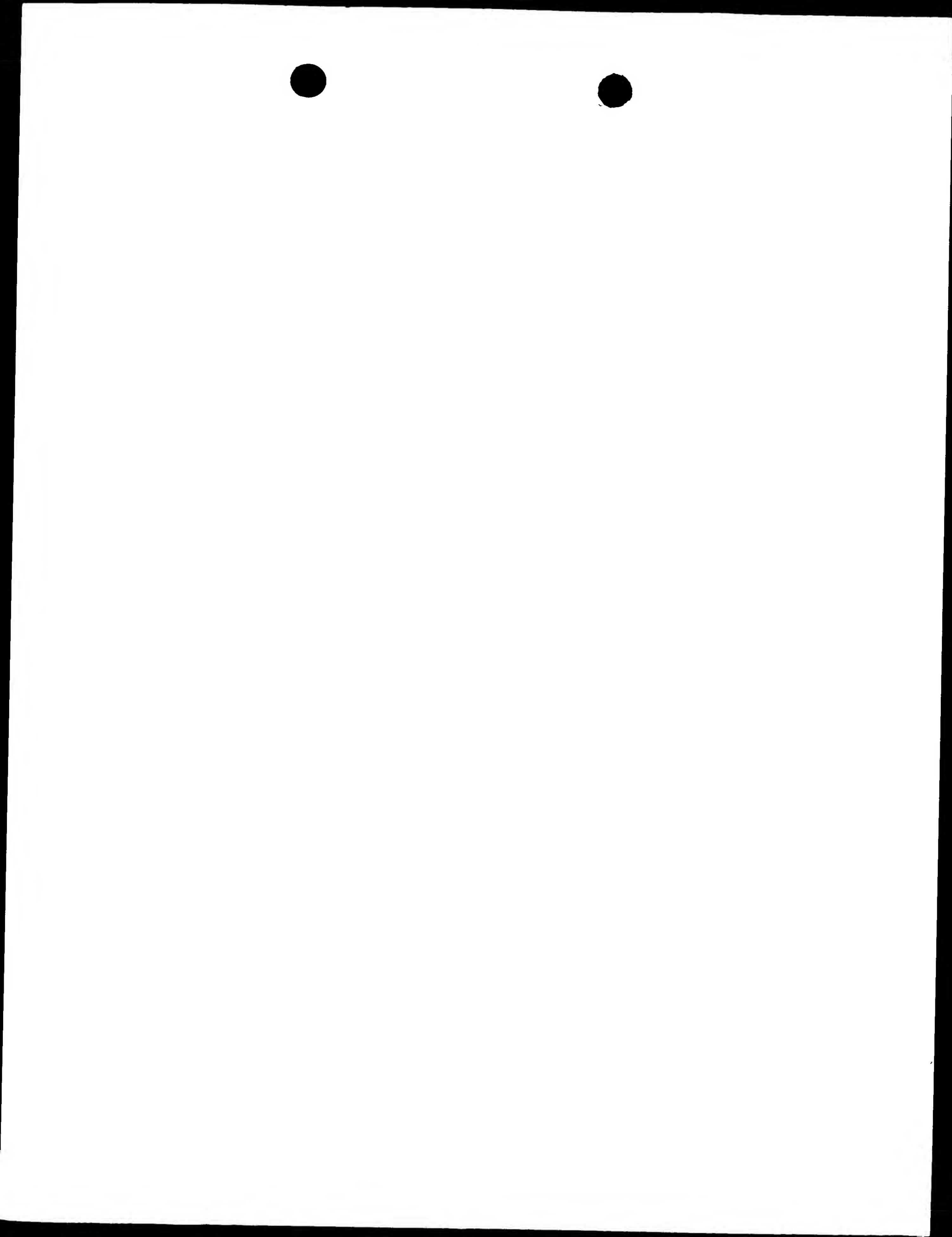
**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International Application No

PCT/CA 00/01114

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9903937	A	28-01-1999	US 5955532 A		21-09-1999
			AU 8486798 A		10-02-1999
			BR 9810601 A		11-07-2000
			CN 1264412 T		23-08-2000
			EP 0996688 A		03-05-2000
<hr/>					
US 5486359	A	23-01-1996	US 6010696 A		04-01-2000
			US 6087113 A		11-07-2000
			US 5733542 A		31-03-1998
			US 5811094 A		22-09-1998
			US 5837539 A		17-11-1998
			AU 669850 B		27-06-1996
			AU 2252492 A		12-01-1993
			CA 2111845 A		23-12-1992
			EP 0592521 A		20-04-1994
			JP 7500001 T		05-01-1995
			WO 9222584 A		23-12-1992
<hr/>					
US 5736396	A	07-04-1998	AU 719098 B		04-05-2000
			AU 4746996 A		14-08-1996
			CA 2211120 A		01-08-1996
			EP 0805853 A		12-11-1997
			JP 10512756 T		08-12-1998
			WO 9623059 A		01-08-1996
			US 5942225 A		24-08-1999
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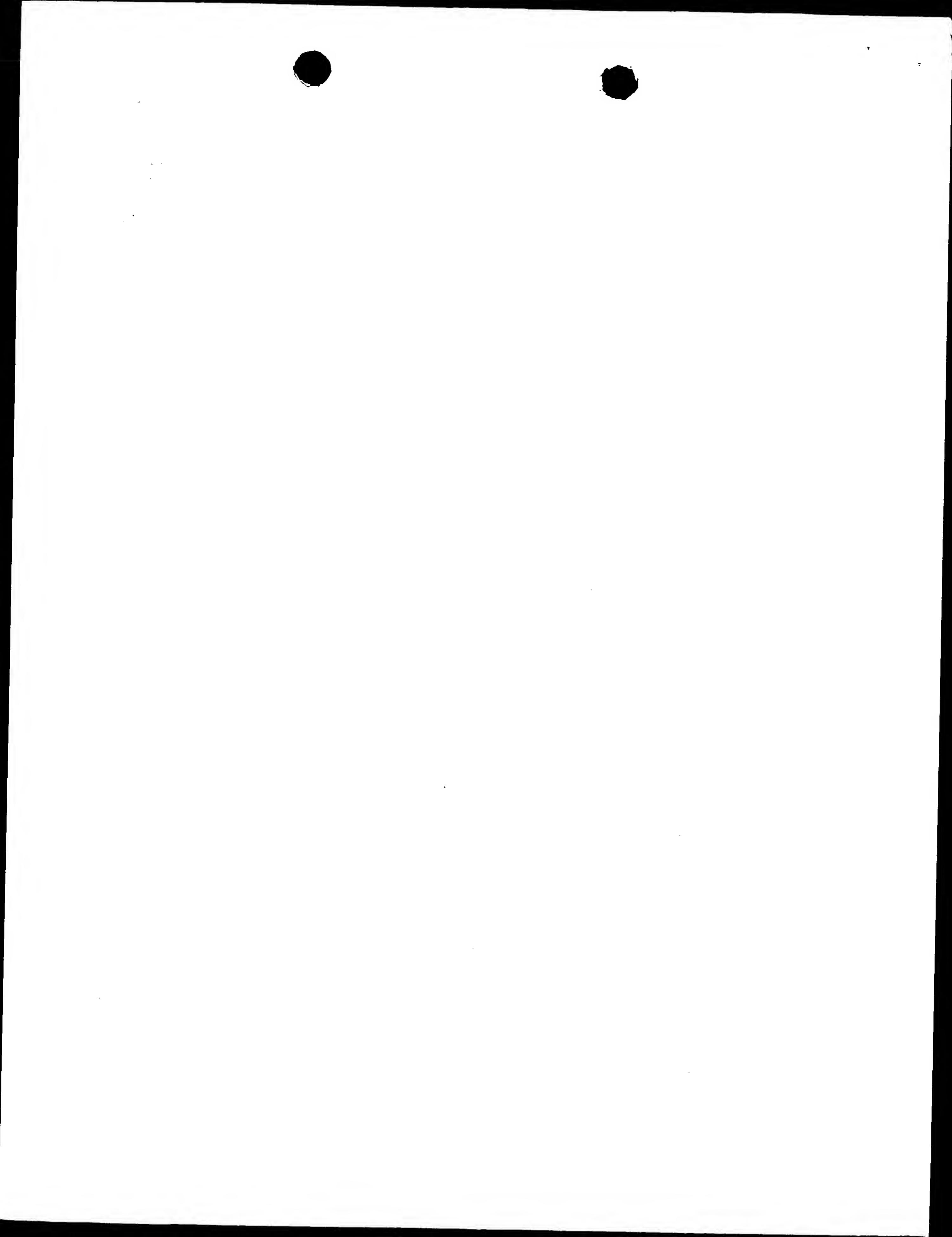


**PATENT COOPERATION TREATY**  
**PCT**  
**INTERNATIONAL PRELIMINARY EXAMINATION REPORT**  
(PCT Article 36 and Rule 70)

Applicant's or agent's file reference  1770-232PCT	<b>FOR FURTHER ACTION</b>	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No.  PCT/CA00/01114	International filing date (day/month/year)  28/09/2000	Priority date (day/month/year)  30/09/1999
International Patent Classification (IPC) or national classification and IPC  A61K35/12		
Applicant  MCGILL UNIVERSITY		
<p>1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of 6 sheets, including this cover sheet.</p> <p><input type="checkbox"/> This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).</p> <p>These annexes consist of a total of sheets.</p>		
<p>3. This report contains indications relating to the following items:</p> <ul style="list-style-type: none"> <li>I    <input checked="" type="checkbox"/> Basis of the report</li> <li>II    <input type="checkbox"/> Priority</li> <li>III    <input checked="" type="checkbox"/> Non-establishment of opinion with regard to novelty, inventive step and industrial applicability</li> <li>IV    <input type="checkbox"/> Lack of unity of invention</li> <li>V    <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement</li> <li>VI    <input type="checkbox"/> Certain documents cited</li> <li>VII    <input type="checkbox"/> Certain defects in the international application</li> <li>VIII    <input type="checkbox"/> Certain observations on the international application</li> </ul>		

Date of submission of the demand  27/04/2001	Date of completion of this report  13.12.2001
Name and mailing address of the international preliminary examining authority:   European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer  Deck, A  Telephone No. +49 89 2399 8432





**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/CA00/01114

**I. Basis of the report**

1. With regard to the elements of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):

**Description, pages:**

1-38                   as originally filed

**Claims, No.:**

1-17                   as originally filed

**Drawings, sheets:**

1/7-7/7               as originally filed

2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

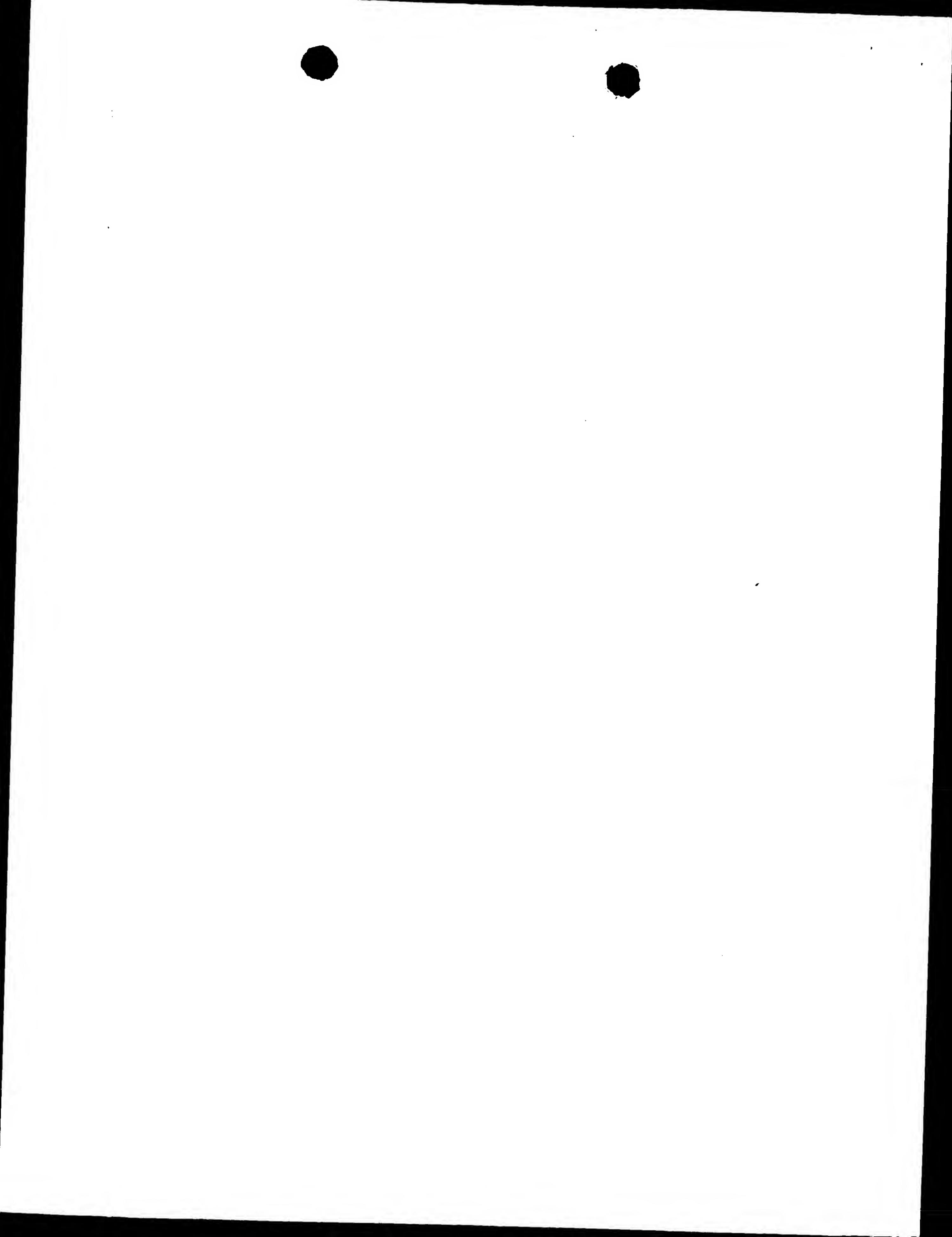
- the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- the language of publication of the international application (under Rule 48.3(b)).
- the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- contained in the international application in written form.
- filed together with the international application in computer readable form.
- furnished subsequently to this Authority in written form.
- furnished subsequently to this Authority in computer readable form.
- The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- the description,           pages:
- the claims,              Nos.:



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/CA00/01114

the drawings, sheets:

5.  This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)): *(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)*

6. Additional observations, if necessary:

**III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

the entire international application.

claims Nos. 1-17.

because:

the said international application, or the said claims Nos. 1-17 relate to the following subject matter which does not require an international preliminary examination (specify):  
**see separate sheet**

the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (specify):

the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

the written form has not been furnished or does not comply with the standard.

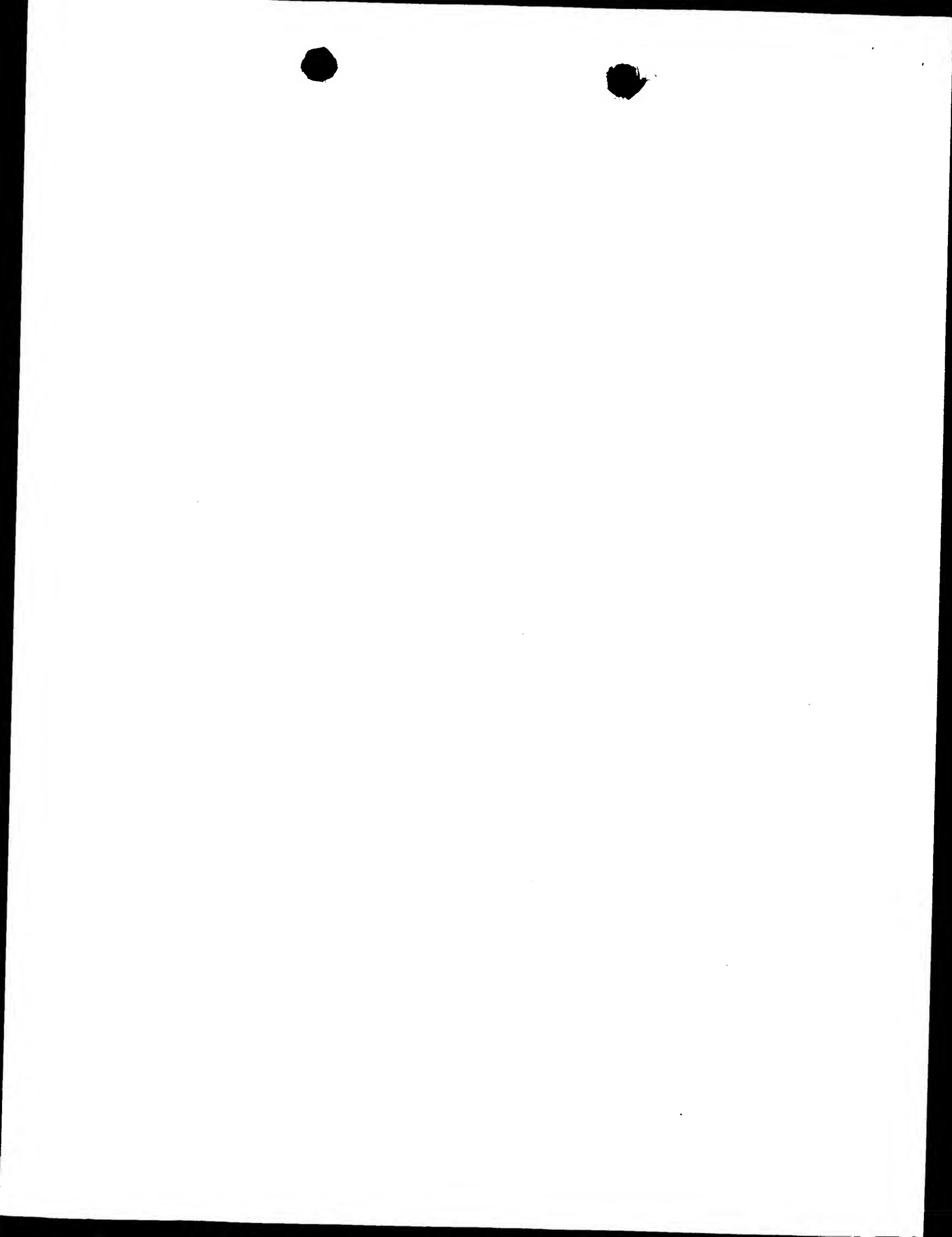
the computer readable form has not been furnished or does not comply with the standard.

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)

Yes: Claims

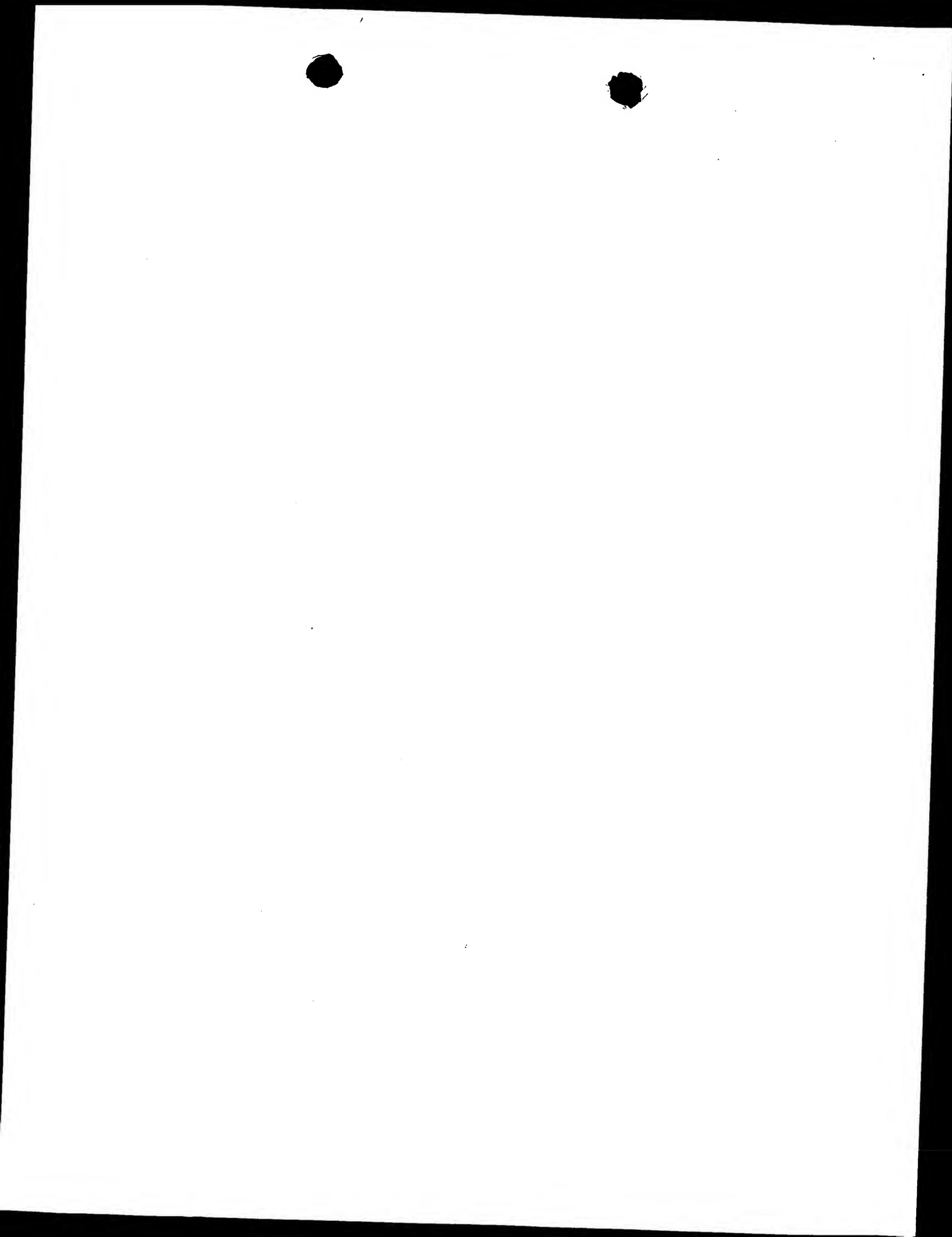


**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/CA00/01114

	No:	Claims	1-17
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-17
Industrial applicability (IA)	Yes:	Claims	
	No:	Claims	see separate sheet

2. Citations and explanations  
**see separate sheet**



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/CA00/01114

**Section III:**

Claims 1-17 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

**Section V:**

1. The following documents are referred to:

D1: WO 99 03973 A (OSIRIS THERAPEUTICS INC ;GORDON STEPHEN L (US); MACKAY ALASTAIR MO) 28 January 1999 (1999-01-28)

D2: TOMITA S ET AL: 'AUTOTRANSPLANTED MESENCHYMAL STEM CELLS IMPROVE FUNCTION AFTER A MYOCARDIAL INFARCTION' CIRCULATION,US,AMERICAN HEART ASSOCIATION, DALLAS, TX, vol. 98, no. SUPPL. 17, 1998, page ABS1036

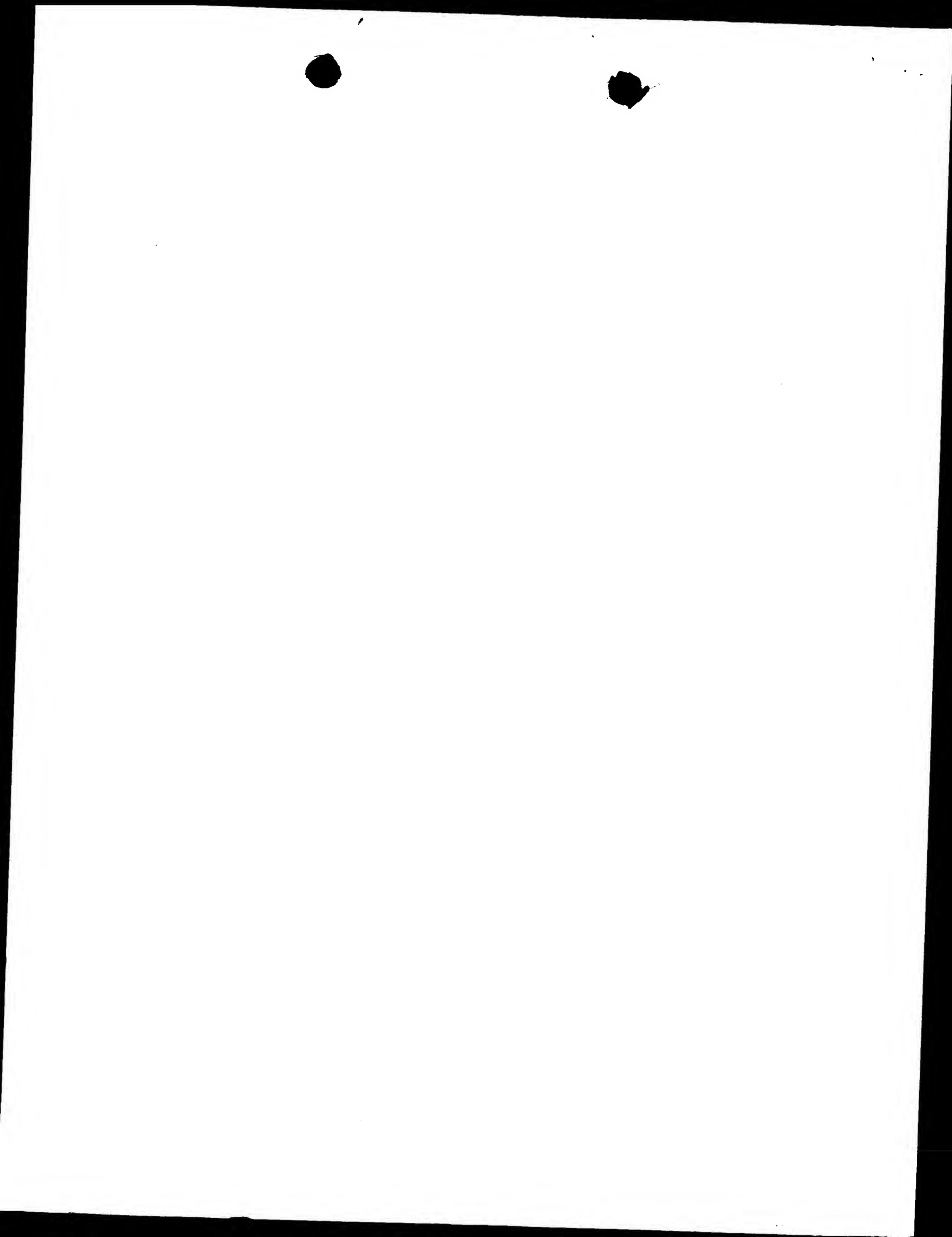
D3: PROCKPO D. ET AL: 'Marrow stromal cells for nonhematopoietic tissues' SCIENCE, vol. 276, 1997, pages 71-74

D4: KESSLER P D ET AL: 'MYOBLAST CELL GRAFTING INTO HEART MUSCLE: CELLULAR BIOLOGY AND POTENTIAL APPLICATIONS' ANNUAL REVIEW OF PHYSIOLOGY,US,ANNUAL REVIEWS, INC., PALO ALTO, CA, vol. 61, 1999, pages 219-242

2. The invention pertains to the use of autologous bone marrow stroma cells (MSCs) to grow new muscle cells in the myocardium of a patient. These bone marrow stroma cells are also known as "mesenchymal stem cells" (see for example D3, page 71, and D4, page 232, second paragraph).

The documents D1 and D2 both disclose the use of autologous MSCs for cardiac muscle regeneration.

D1 further describes the MSCs to be implanted as being marked to trace their fate as they proliferate and differentiate following their introduction in the heart (e.g.



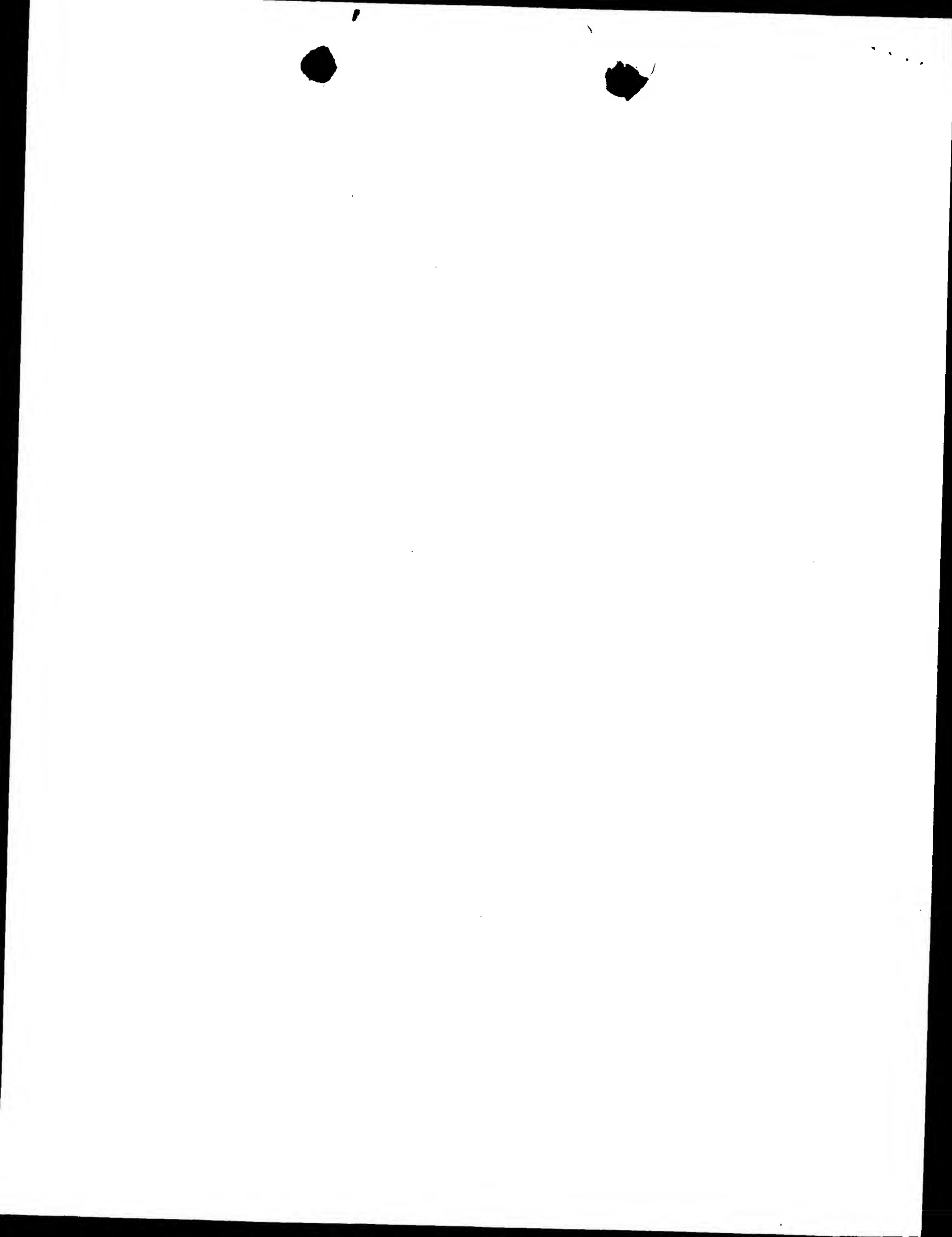
**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/CA00/01114

page 9, lines 14-16).

Therefore, the application does not meet the requirements of Article 33 (2) and (3) PCT as the subject-matter of claims 1-17 appears to be neither novel, nor inventive.

3. For the assessment of the present claims 1-17 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.



(19) World Intellectual Property Organization  
International Bureau



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WO 01/22978 A2

(51) International Patent Classification<sup>7</sup>: A61K 35/12    (74) Agents: QUINN, Jennifer et al.; Swabey Ogilvy Renault, 1981 McGill College Avenue, Suite 1600, Montréal, Québec H3A 2Y3 (CA).

(21) International Application Number: PCT/CA00/01114

(22) International Filing Date: 28 September 2000 (28.09.2000)

(25) Filing Language: English

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(30) Priority Data: 60/156,700    30 September 1999 (30.09.1999) US

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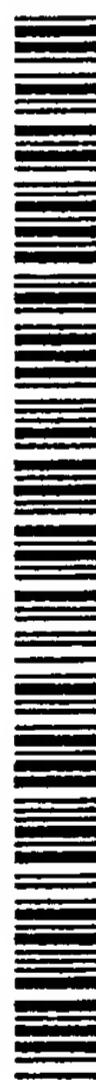
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**Published:**

— Without international search report and to be republished upon receipt of that report.

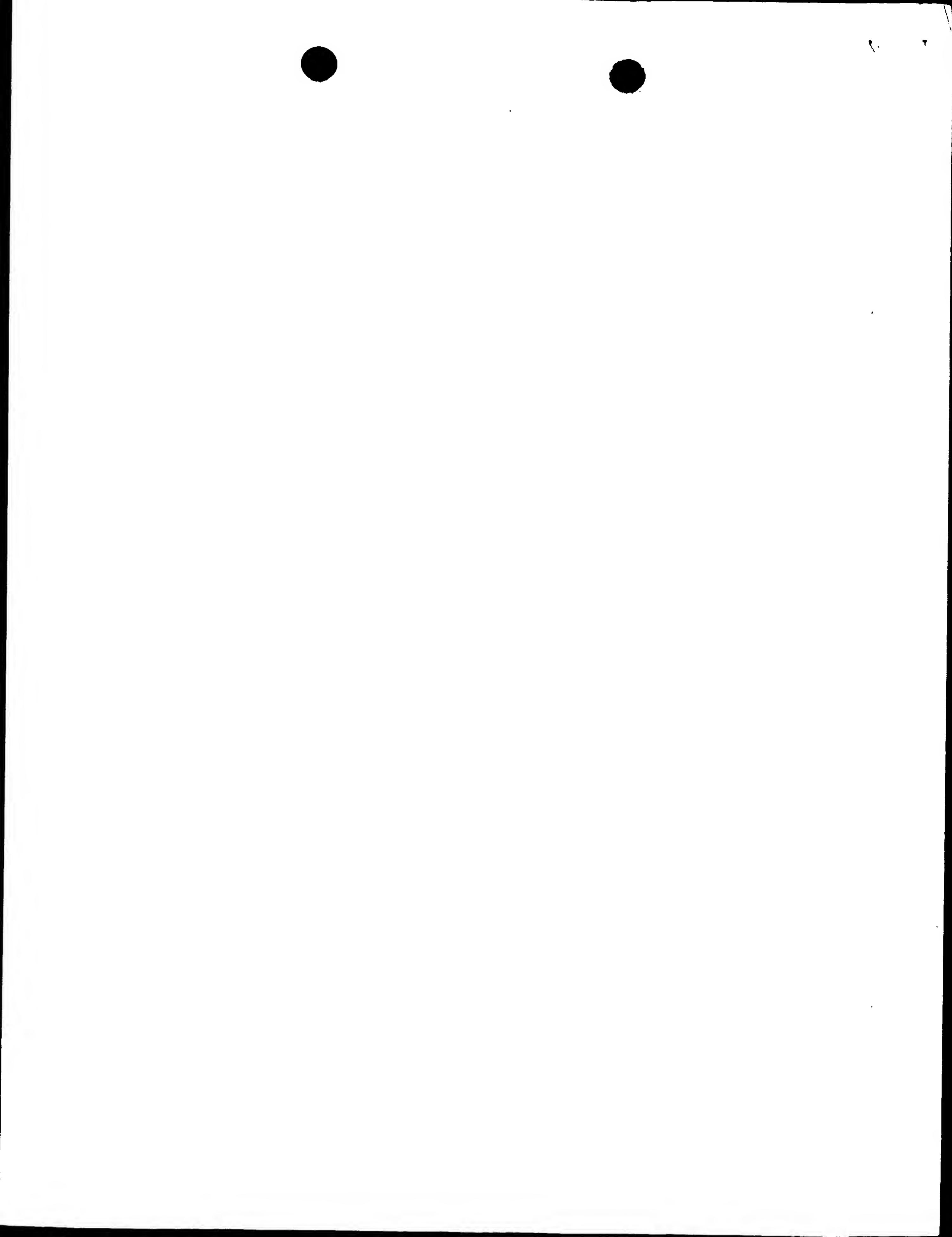
For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

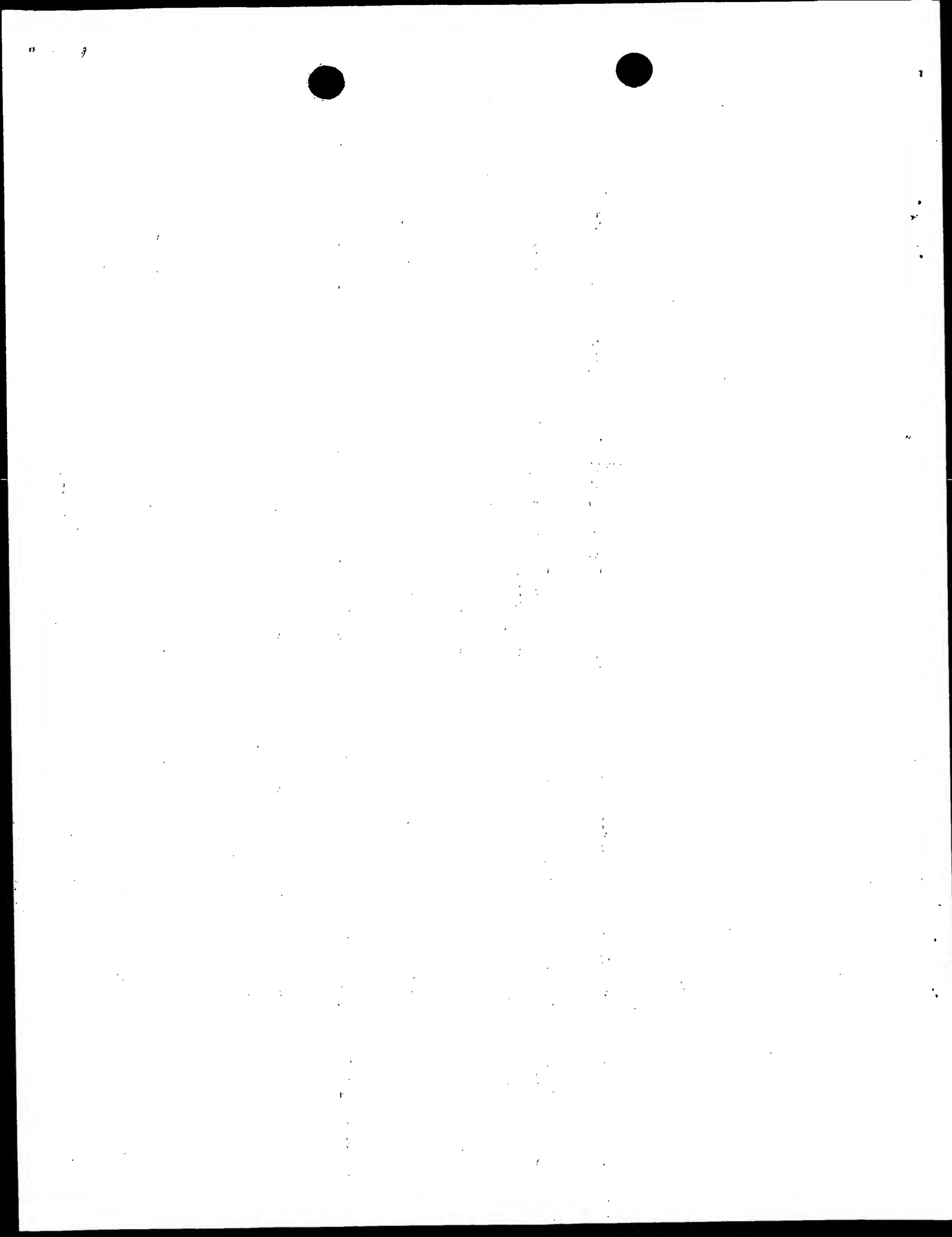


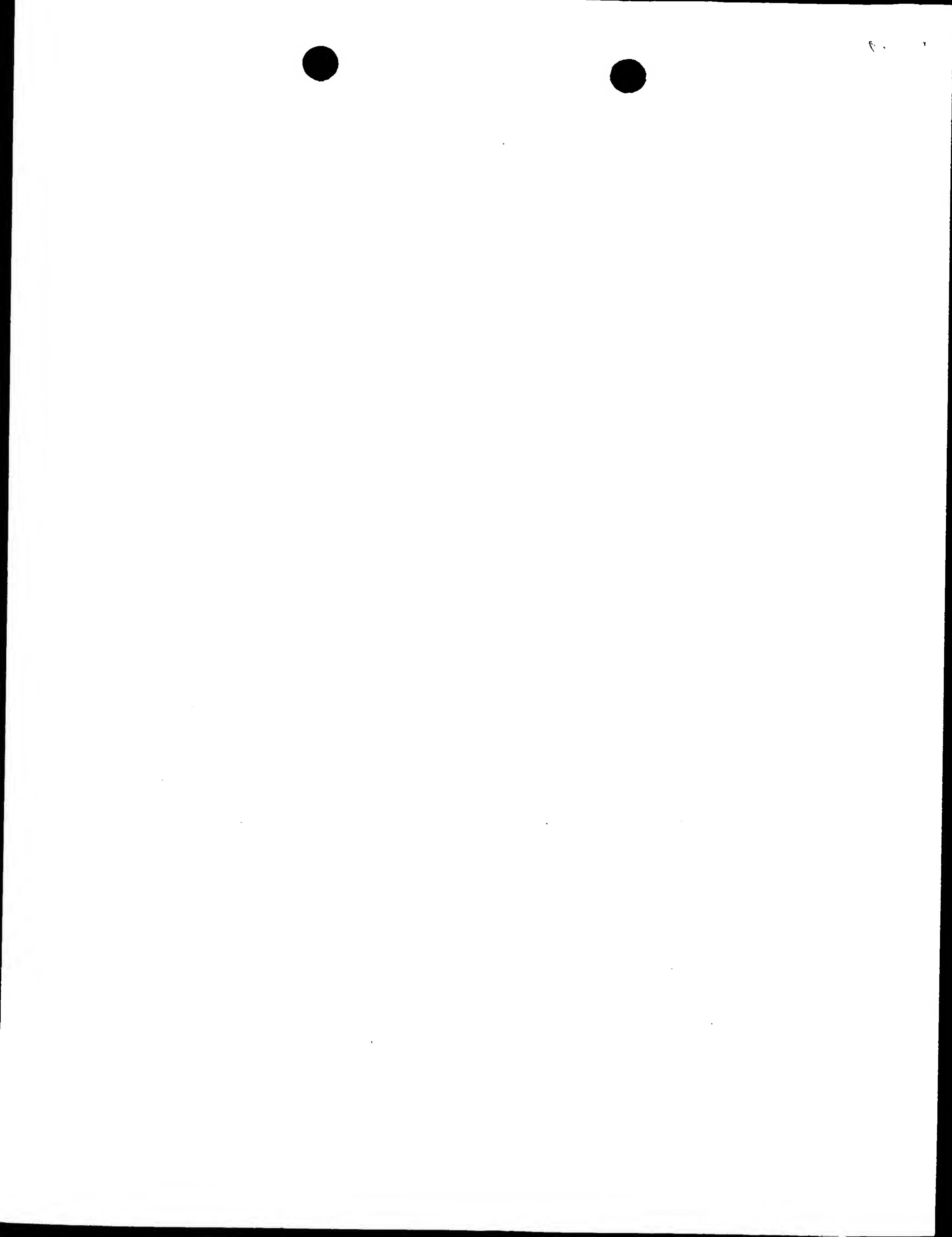
WO 01/22978 A2

(54) Title: AUTOLOGOUS MARROW STEM CELL (MSC) TRANSPLANTATION FOR MYOCARDIAL REGENERATION

(57) Abstract: The present invention relates to a method of improving cardiac function in a patient with heart failure without eliciting an immune response and without sacrificing the patient's skeletal muscle; which comprises the step of transplanting autologous bone marrow stroma cells (MSCs) into said patient's myocardium to grow new muscle fibers. The method may further comprise the step of using cell labeling technique to confirm survival and differentiation of implanted MSCs, and to identify said MSCs phenotype by both morphology and molecular markers. The method may further comprise examining the effects of the micro-environment of implanted MSCs on their differentiation and phenotype expression. The method may further comprise examining functional contribution of MSCs implanted into an ischemic segment of the myocardium.







JCIS Rec'd PCT/PTO 01 APR 2002

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- 1 -

AUTOLOGOUS MARROW STEM CELL (MSC) TRANSPLANTATION  
FOR MYOCARDIAL REGENERATION

TECHNICAL FIELD

The invention relates to autologous bone marrow stroma cells (MSCs) and the use of these cells in improving cardiac function in patients with heart failure. In particular, the present invention relates to the transplantation of MSCs into the myocardium to grow new muscle fibers. The specific aims of the invention include:

5      1) using cell labeling techniques to confirm the survival and differentiation of implanted MSCs, and to identify their phenotype by both morphology and molecular markers;

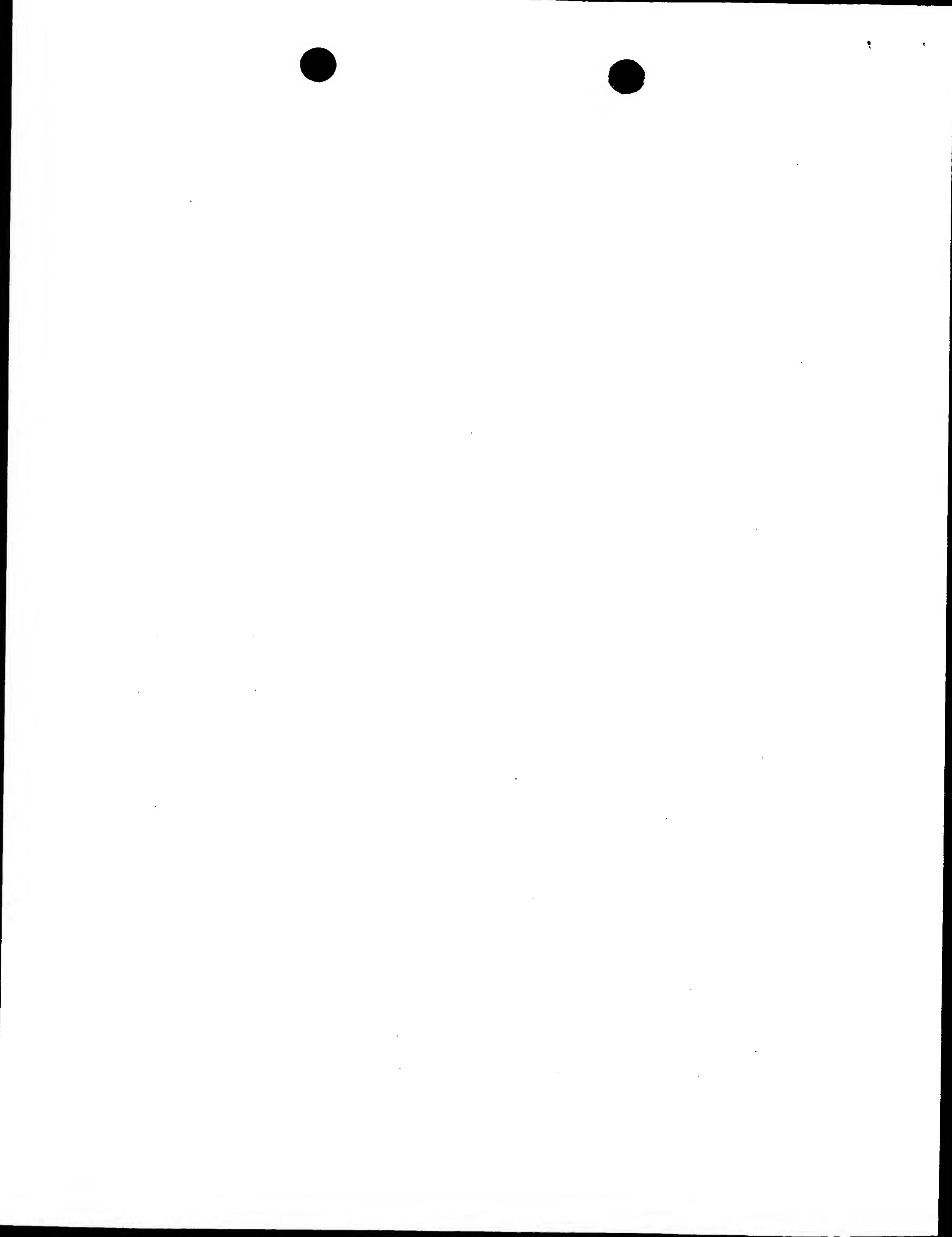
10     2) examining the effects of the micro-environment of the implanted MSCs, on their differentiation and phenotype expression; and 3) the functional contribution of MSCs when implanted into an ischemic segment of a myocardium.

15

BACKGROUND ART

Heart failure is both common and deadly. In Canada, approximately 50,000 new cases of heart failure are diagnosed, and more than 300,000 patients currently suffer this condition. Heart failure is the only major cardiovascular disorder that is increasing in incidence and mortality at present, and in Class IV patients, one year mortality approaches 50% in spite of advances in drug therapy in recent years (L'enfant, C: Fixing the failing heart. Circulation 1997; 95:771-772).

Artificial heart and mechanical cardiac assist are largely used as a bridge to transplantation today, and they still face formidable difficulties of thromboembolic complications and suitable energy source. Cardiac transplantation can have dramatic improvements in terminal

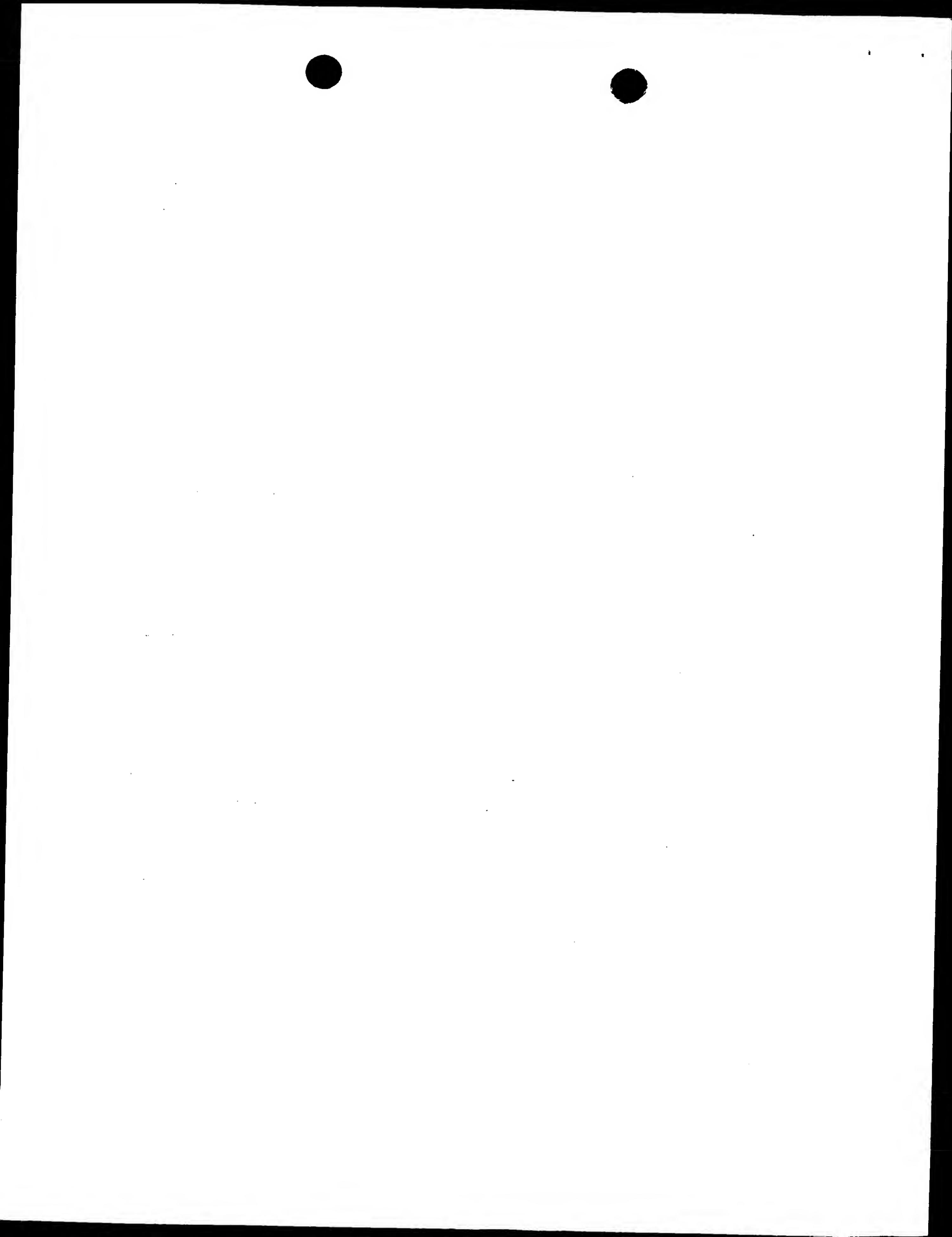


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heart failure patients, but its availability is severely restricted by the donor availability, as well as by the complications associated with immunosuppression. These approaches are also very expensive, straining the limited 5 health care resources.

One exciting new approach in recent years is tissue engineering, in which various cells are cultured *in vitro* over biodegradable polymer scaffolds to create a 3-dimensional construct *in vitro*, which can then be implanted 10 to replace damaged tissues or organs. Upon the absorption of a biodegradable scaffold *in vivo*, these replacement tissues would not require immunosuppression if autologous donor cells were used for tissue engineering. Advances are being made in constructing cardiovascular structures, such 15 as arteries and cardiac valves. Attempts are also being made to engineer 3-dimensional myocardial tissue blocks by seeding cardiac myocytes on a 3-dimensional scaffold, and cultured in rotating bioreactors. Without concomitant creation of a coronary vascular system within these 20 constructs, however, such tissue engineered myocardium cannot be used therapeutically *in vivo*, as they will suffer rapid ischemic necrosis.

Such difficulties described above may be circumvented by tissue engineering neomyocardium *in vivo*. 25 As is well known, cardiomyocyte loss from myocyte necrosis and apoptosis plays an important role in the initiation and progression of heart failure (Olivetti G, Abbi R, Quaini F, et al.: Apoptosis in the failing human heart. N Engl J Med 1997;336:1131-1141). Cellular cardiomyoplasty is a potential 30 future therapy for heart failure in which donor cells with the potential to differentiate into cardiac myocytes are implanted into the damaged myocardium in order to regenerate new muscle fibers (Chiu RC-J, Zibaitis A, Kao



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RL: Cellular cardiomyoplasty: Myocardial regeneration with satellite cell implantation. Ann Thorac Surg 1995; 60:12-18). To date, a number of donor cells have been studied by various investigators, and are summarized below.

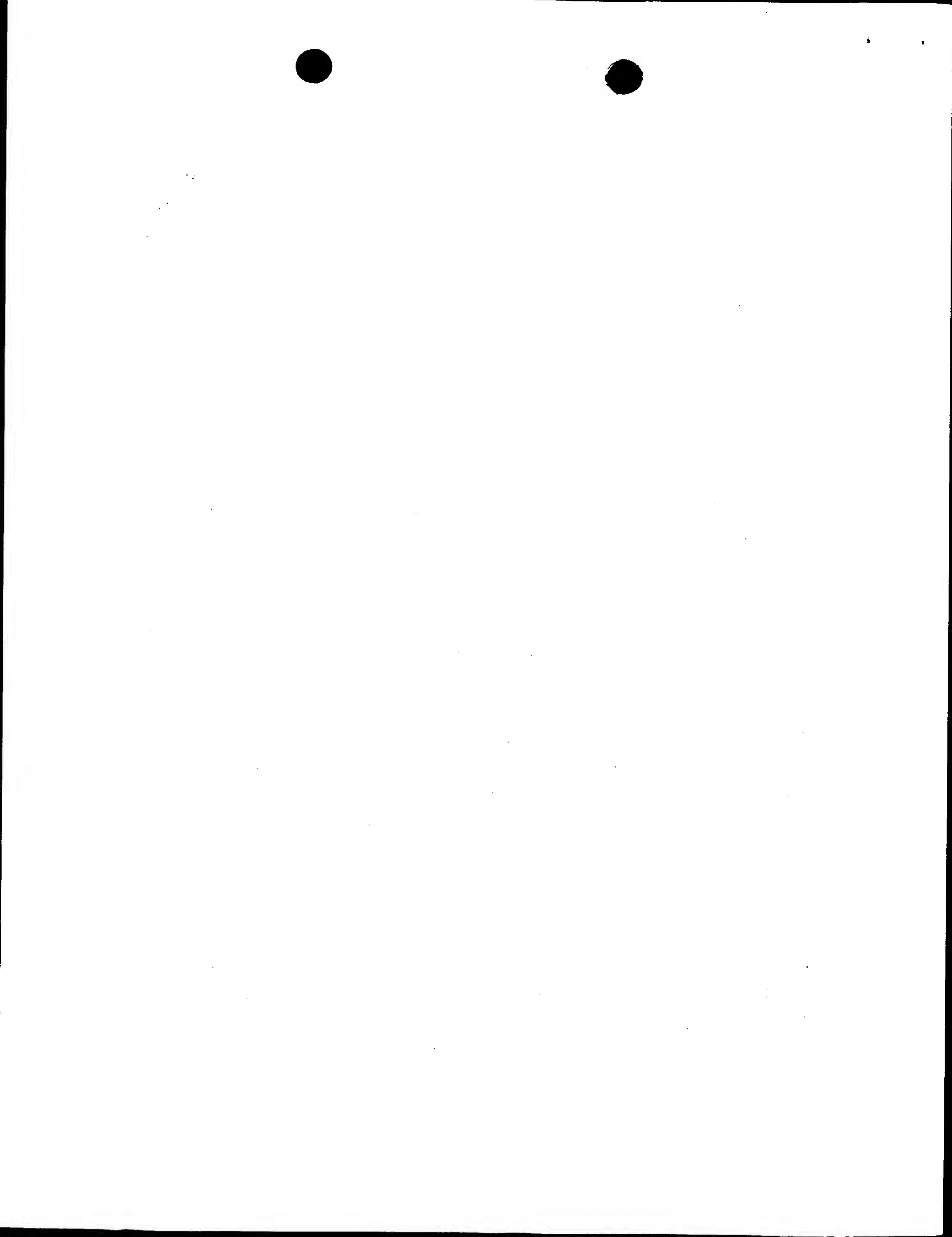
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#### A. Fetal Cardiomyocytes

Differentiated fetal cardiomyocytes retain a capacity for proliferation. Both in rodent and in canine models, fetal cardiomyocytes implanted into the myocardial wall of adult animals have been shown to be successfully engrafted, and develop into cells which are morphologically and functionally indistinguishable with the native cardiac myocytes within the recipient heart. They form gap junctions which should allow them to be depolarized and contract synchronously as a syncytium (Soonpaa MH, Koh GY, Klug MG, et al.: Formation of nascent intercalated disks between grafted fetal cardiomyocytes and host myocardium. Science 1994; 264: 98-101). By using fetal cells, however, the problem of donor cell availability becomes more formidable and the ethical issues more complex. Unlike for the use of fetal cells for treatment of neurological diseases, such as Parkinson's disease, in which engraftment of small numbers of cells may be adequate for therapeutic effect, fetal cardiomyocyte transplantation is likely to require millions of new cells to be efficacious, and continued proliferation of engrafted myocytes cannot be expected to expand the population, once they are removed from the donor embryos.

30       B. Embryonic Stem Cells

Klug et al. transfected a transgene that confers resistance to a toxic drug into embryonic stem cells under the control of a cardiac specific promoter (Klug M.G.,

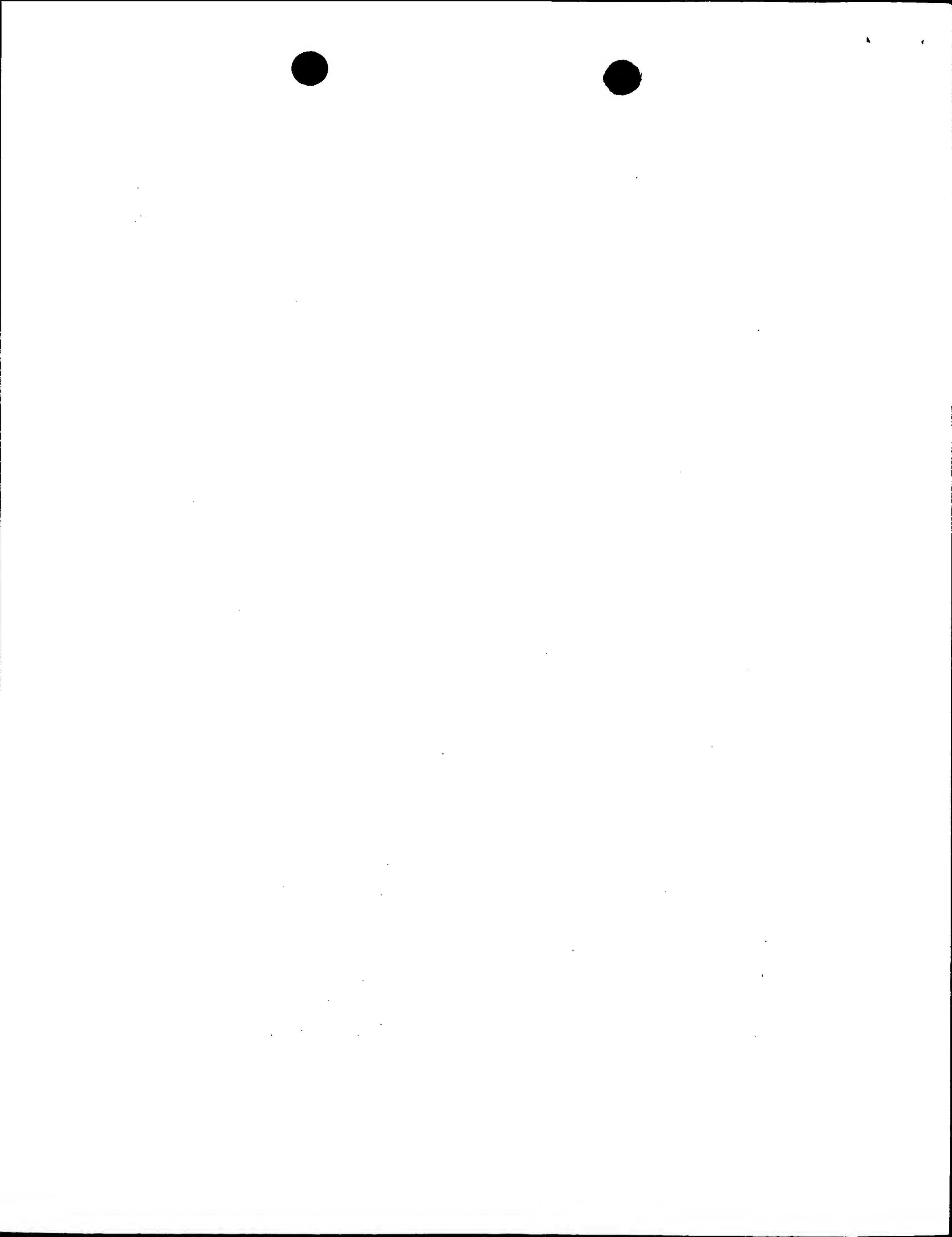


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Soonpaa M.H., Koh G.Y., Field L.J. Genetically selected cardiomyocytes from differentiating embryonic stem cells form stable intracardiac grafts. J. Clin. Invest. 1996; 98:216-24). When embryoid bodies derived from these stably transfected embryonic stem cells were exposed to the toxic antibodies, only cardiac myocytes survived. These cells were harvested and injected into the myocardial wall of the adult mice, where they engrafted and formed appropriate cell-to-cell junctions, i.e. intercalated discs with desmosomes and gap junctions, with host cardiomyocytes while maintaining a morphologically differentiated state. In principle, this strategy would allow for generating large numbers of donor myocytes. However, the ethical issues of using embryonic stem cells are currently hotly debated.

#### C. Modified Adult Cardiomyocytes and Myoblast Cell Lines

The adult cardiomyocytes are generally believed to be terminally differentiated and thus unable to proliferate. It has been previously reported that adult cardiomyocytes obtained from biopsy can be induced to proliferate *in vitro*, while retaining some phenotypic characteristics of the cardiac myocytes, and they can be successfully engrafted into the myocardium (Li RK, Jia ZQ, Weisel RD et al. Cardiomyocyte transplantation improves heart function. Ann. Thor. Surg. 1996; 62:654-660). It was shown that such cells implanted into an ischemic myocardium could improve ventricular function. These findings, however, require independent confirmation, more precise identification of cellular phenotype, and assurance against oncogenicity of the transformed cardiac myocytes before they can be considered for clinical use.

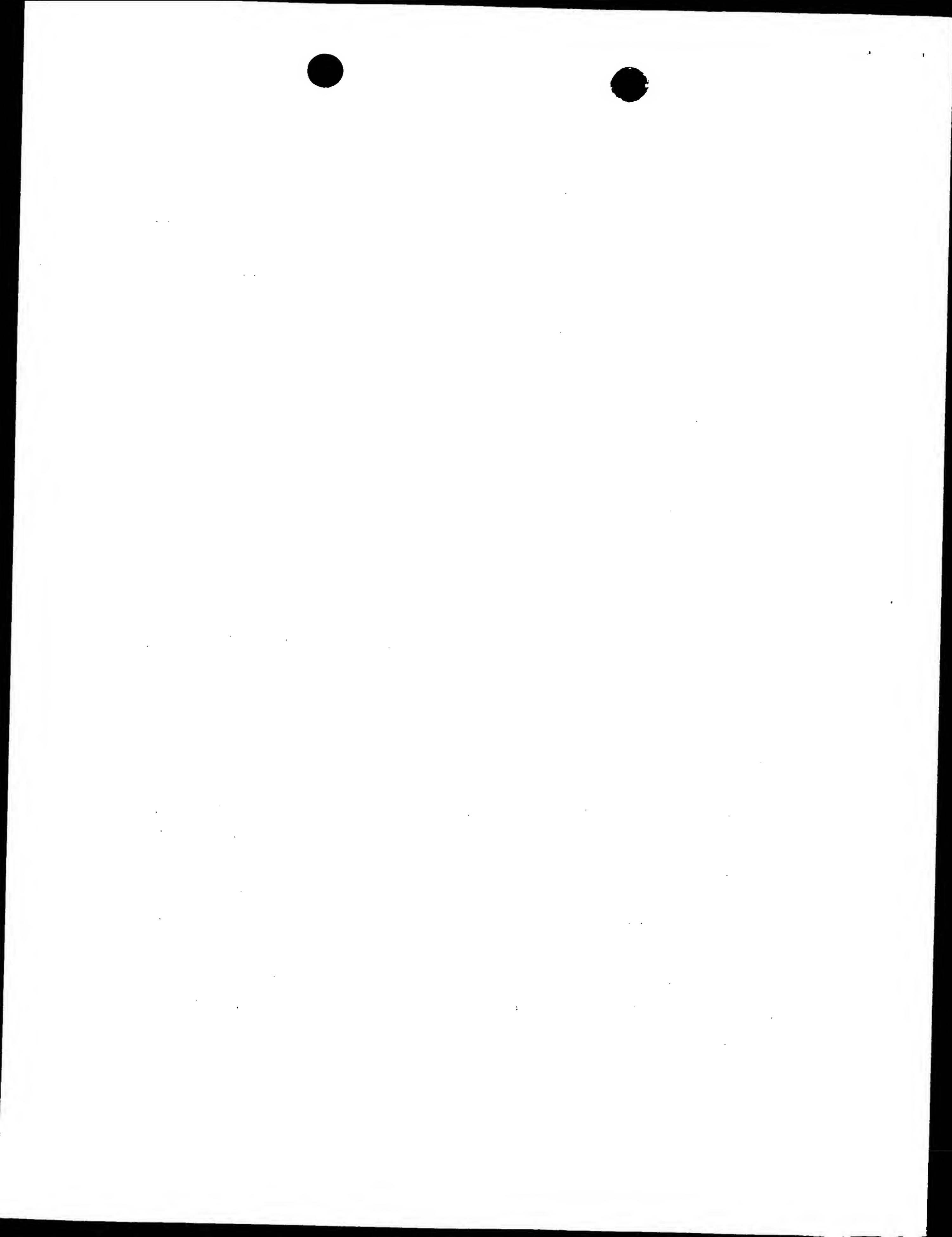


- 5 -

Robinson et al. and other investigators implanted cells from established cell lines, such as C2 C12 cells which were originally derived from skeletal myoblasts (satellite cells) (Robinson SW, Cho PW, Levitsky HI et al.: 5 Arterial delivery of genetically labeled skeletal myobalsts to the murine heart: Long-term survival and phenotypic modification of implanted myoblasts. Cell Transplantation 5:77-91, 1996). There is evidence that such cells, in spite of their origin from skeletal muscle, may 10 transdifferentiate in the heart acquiring certain phenotypic characteristics of a cardiac myocyte, such as the expression of Connexin-43 and the formation of desmosomes at cell junctions. Although it is very convenient to use established cell lines which can be 15 purchased from suppliers, the concern of oncogenicity upon transplantation *in vivo*, and the need for immunosuppression may limit their application in clinical therapy.

#### D. Adult Skeletal Myoblasts (Satellite Cells)

20 The feasibility of implanting autologous myoblasts (satellite cells) harvested from the adult skeletal muscle has also been explored (Chiu, RCJ, Zibaitis A, Kao L: Cellular cardiomyoplasty: Myocardial regeneration with satellite cell implantation. Ann. Thorac. Surg. 60:12-18, 25 1995). Using cell labeling techniques and phenotype specific antibodies, strong evidence has been presented that these myoblasts can undergo milieu-dependent transdifferentiation, and develop into striated muscle fibers with slow myosin heavy chains, as well as 30 intercalated discs expressing Connexin-43. Taylor et al. confirmed that such engulfed satellite cells show ultrastructural features similar to immature cardiac myocytes, and when the implantation took place within a



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cryo-injured myocardium, cellular cardiomyoplasty could improve both the systolic and diastolic functions of such hearts (Taylor, D.A.; Atkins B.Z., Hungspreugs P., et al.: Regenerating functional myocardium: Improved performance 5 after skeletal myoblast transplantation. Nat. Med. 1998; 4:929-933). Nevertheless, it would require sacrificing the patient's skeletal muscle, and the concern that the number of satellite cells in the skeletal muscle, as well as the satellite cells' mitotic potential may decrease with age. 10 The optimal "conditions" for satellite cells to transdifferentiate into cardiomyocytes have not been clearly defined, and the molecular mechanisms of milieu-dependent differentiation remain unknown.

Thus, it would be highly desirable to identify a 15 suitable cell type for use in effecting *in vivo* myocardial regeneration.

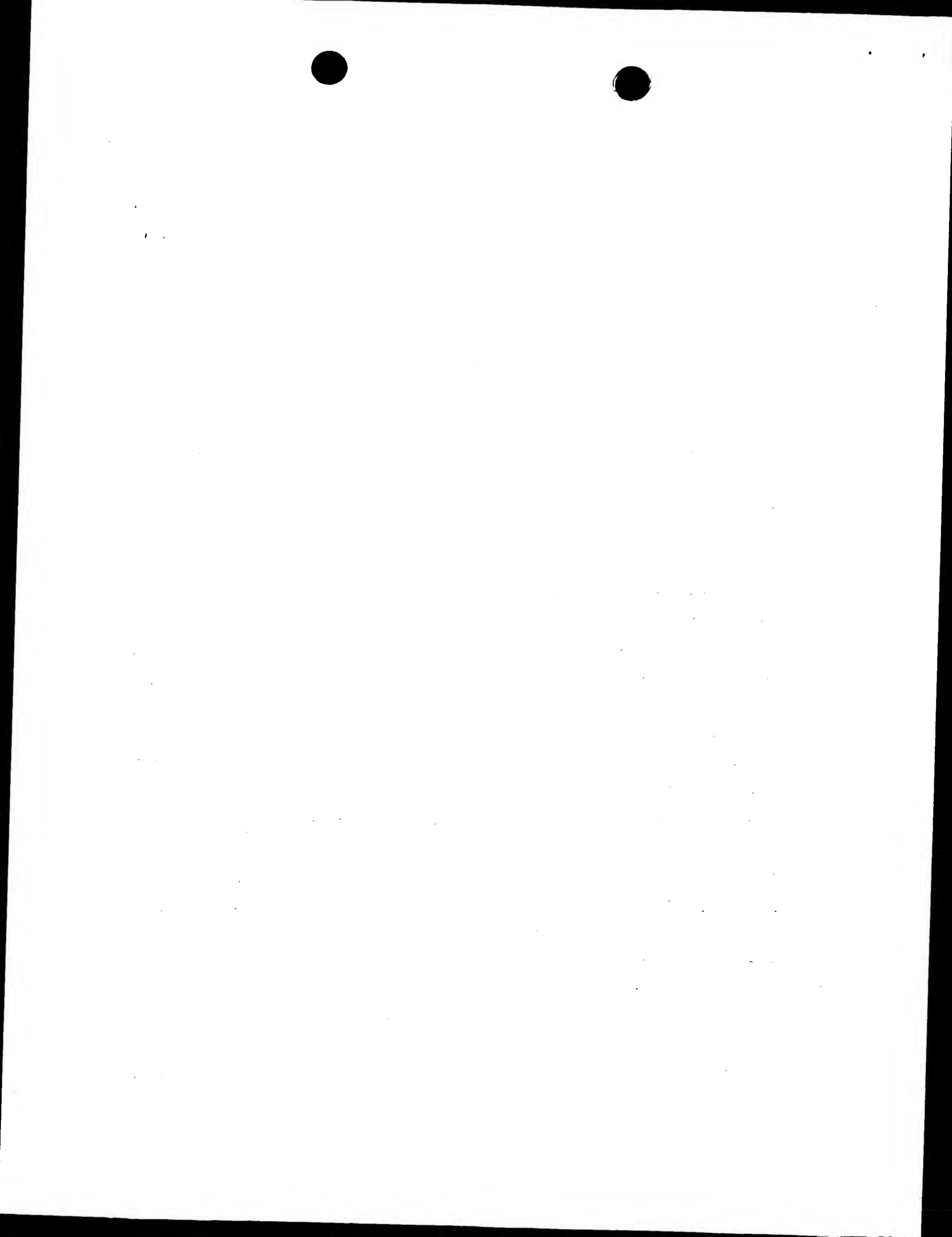
It would be further desirable to be provided with means to perform myocardial implantation without eliciting an immune response and without sacrificing the patient's 20 skeletal muscle.

#### DISCLOSURE OF THE INVENTION

One aim of the present invention is to provide means to perform myocardial implantation without eliciting an 25 immune response and without sacrificing the patient's skeletal muscle.

Another aim of the present invention is to use autologous MSCs in myocardial implantation to improve cardiac function.

30 Another aim of the present invention is to use autologous MSCs in myocardial implantation to effect the growth of new muscle fibers.



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Another aim of the present invention is to provide means for using a cell labeling technique to confirm the survival and differentiation of the implanted MSCs, and to identify MSCs phenotype by both morphology and molecular markers.

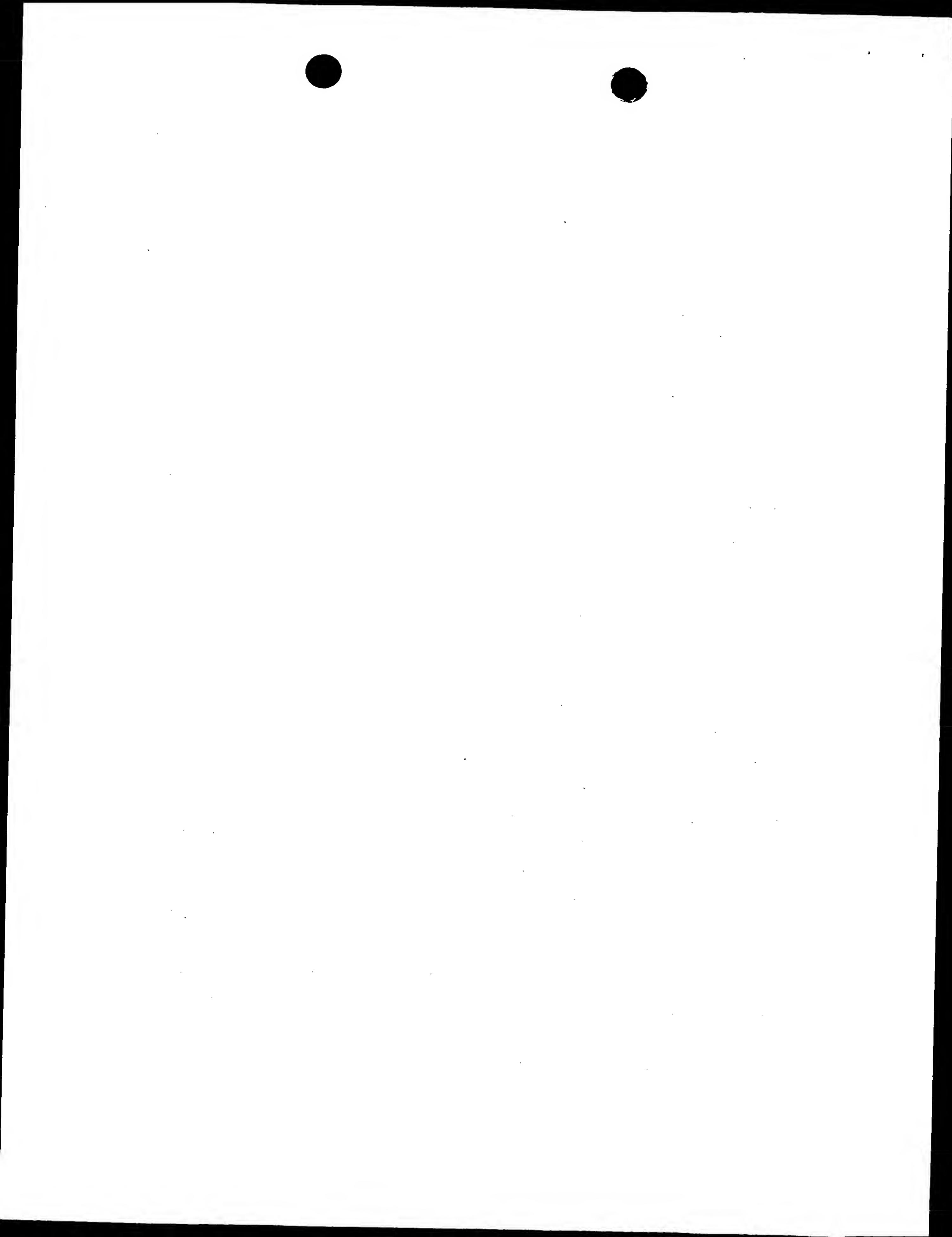
Another aim of the present invention is to provide means to examine the effects of the micro-environment of the implanted cells, on their differentiation and phenotype expression.

Another aim of the present invention is to provide means to examine the functional contribution of MSCs when they are implanted into an ischemic segment of the myocardium.

Another aim of the present invention is to provide a means for using autologous marrow stem cells for improving cardiac function, wherein said autologous marrow stem cells are introduced *in situ* into a myocardium.

The bone marrow stroma micro-environment is a complex network of cells and extracellular matrix, which maintains the hematopoietic system throughout the life of the individual. Hematopoietic cells are found in the spaces between the marrow stroma. The marrow stroma cells (MSCs) are functionally defined as capable of supporting hematopoiesis (Lichtman MD: The relationship of stromal cells to hemopoietic cells in marrow. In *Long Term Bone Marrow Culture*, pp. 57-96, DG Wright, JS Greenberger (eds), Alan R. Liss, New York, 1984), but lacking hematopoietic determinants (e.g., CD45, CD34, CD41, CD14, T- or B-cell markers and Mac-1).

Recent evidence showed that MSCs play a role in the "mesengenic process" for self-repair (Caplan AI: The mesengenic process. Clinics Plast Surg 1994;21:429-435). The body has developed two major strategies for tissue

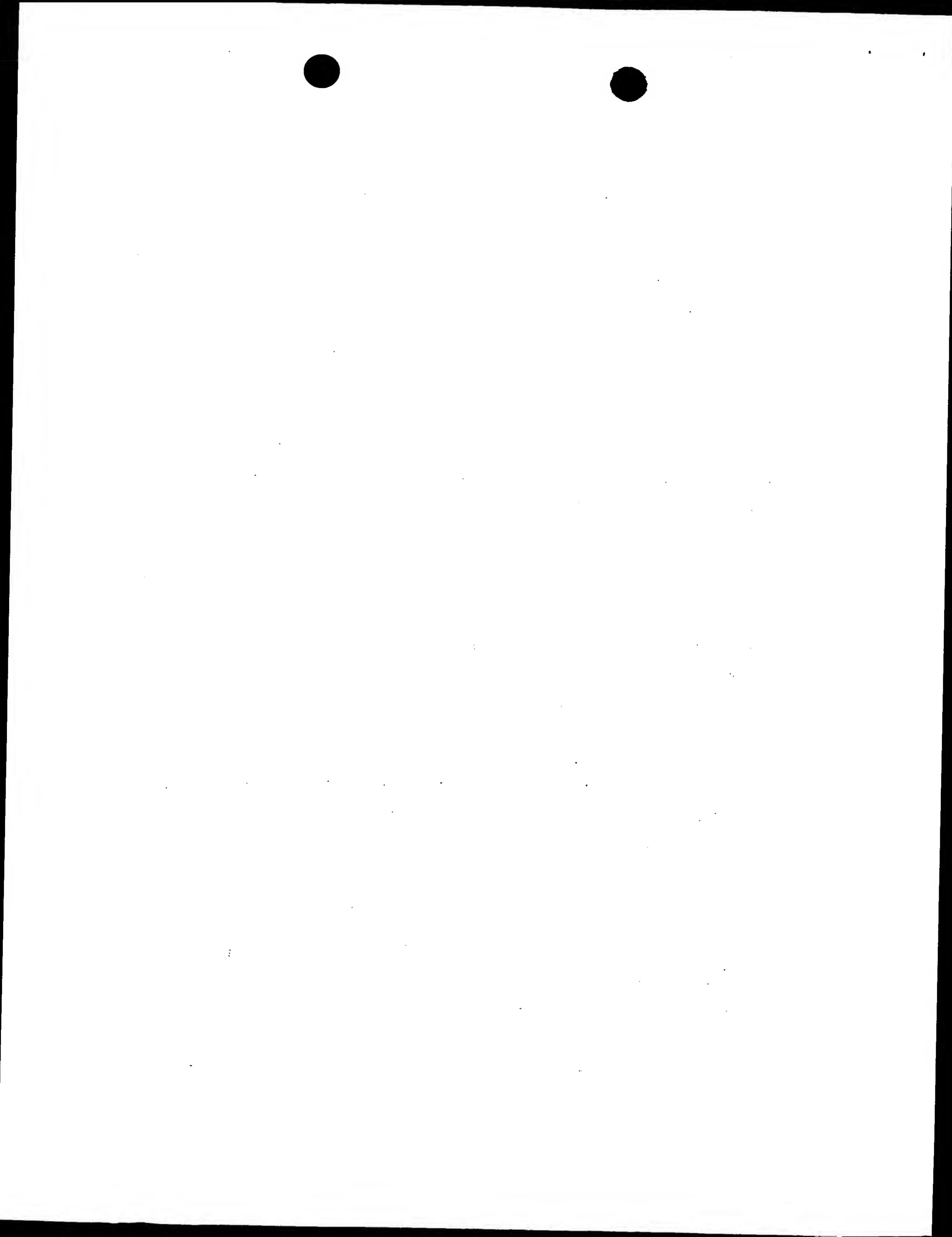


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replacement and renewal. The first way the body attempts cell repair is predicated on the remaining proliferative capacity of differentiated, functioning cells, such as hepatocytes and endothelial cells. The second way is by 5 their regeneration from residual cycling stem cells. An example in this category is the blood cells. All cells of the hematopoietic lineage are derived from a limited number of self-renewing multi-potent cells which respond to the appropriate - cytokines and growth factors for 10 differentiation.

It is now clear that bone marrow also contains cells that meet the criteria for stem cells of non-hematopoietic tissue. These cells are referred to as mesenchymal stem cells, because of their ability to differentiate into cells 15 that can roughly be defined as mesenchyma. These cells are also known as marrow stroma cells (MSCs), because they appear to arise from the supporting structures found in bone marrows.

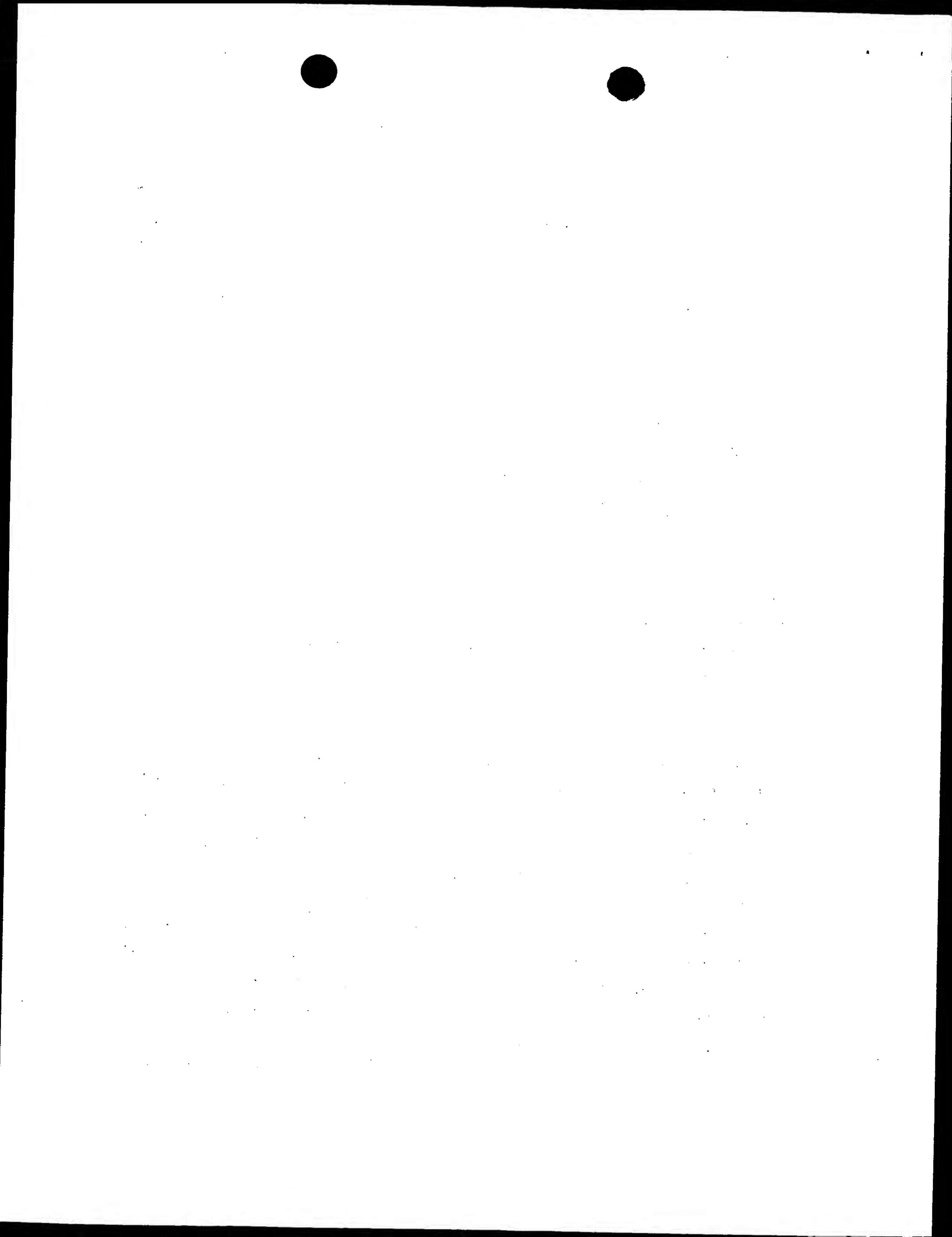
The presence of stem cells for non-hematopoietic 20 cells in bone marrow has been suggested. In addition, the possibility that bone marrow might be the source of fibroblasts that deposit collagen fibers as part of the normal process of wound repair has been suggested, although not fully substantiated (Friedenstein A.J. et al. in Exper. 25 Hematol. 1976; 4:276). According to the study of Freidenstien et al. samples of whole bone marrow were placed in plastic culture dishes and after about 4 hours, poured off the cells that were non-adherent to the dish, which in effect removed most of the hematopoietic stem 30 cells and their progeny. After passage several times in culture, the adherent cells became more uniformly spindle-shaped in appearance. These cells illustrated the ability to differentiate into colonies that resembled small



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deposits of bone or cartilage. Further studies confirmed that MSCs isolated by the procedure of Friedenstein et al. were multi-potential, and readily differentiated into osteoblasts, chondroblasts, adipocytes, and myoblasts. It 5 was further demonstrated that the MSCs, even after 20 to 30 cell doublings in culture were able to maintain their characteristics as multi-potential stem cells, capable of differentiating into various mesenchymal cells listed above.

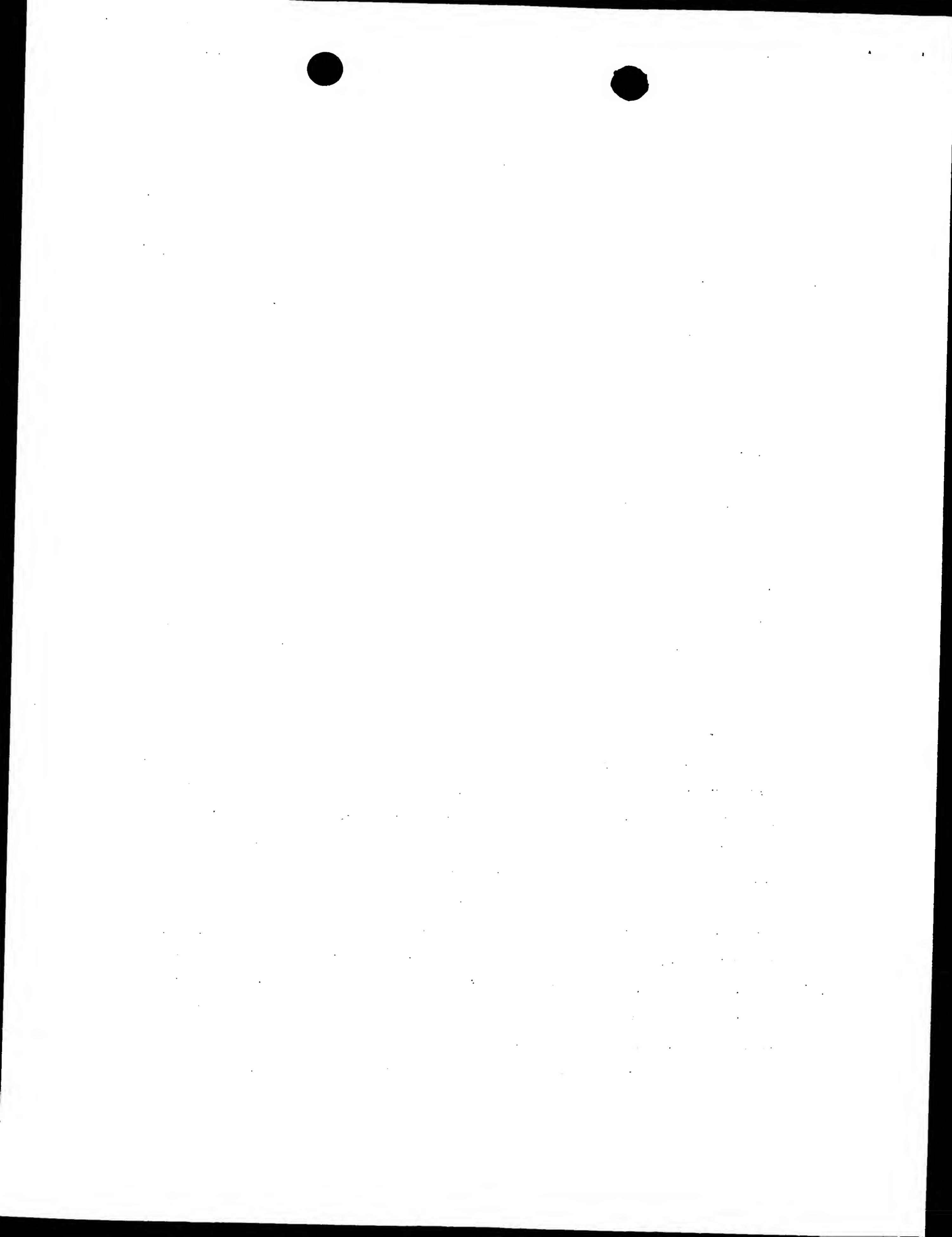
10 Pereira R.F. et al. (Pereira R.F., Halford K., O'Hara M.D. et al.: Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. Proc. Natl. Acad. Sci. 1995; 92:4857-4861) isolated MSCs using the technique of 15 Friedenstein et al. from transgenic mice expressing a mutated collagen gene, which could be used as a cell marker by producing mutated Type I collagen molecules. These MSCs were injected intravenously into mice without this gene marker. After one week, few of the donor cells were found 20 in the recipient mice. At one and five months, however, the donor cells accounted for 1.5 to 12% of the differentiated cells in bone, cartilage, and lung in addition to marrow and spleen. It appeared that the donor MSCs first replaced a portion of the MSCs in the bone marrow of the recipient 25 mice. The MSCs then participated in a normal biological cycle in which MSCs in the bone marrow served as a continuing source of progenitor cells for a variety of mesenchymal tissues in the body. These findings suggested that the progeny of MSCs acquired the phenotype of 30 different target tissues, either before they left the marrow, or after they have entered the micro-environment of the tissue itself through "milieu-dependent differentiation". Thus, it was postulated that MSCs



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participate in the "mesengenic process" which continues throughout life (Caplan A.I.: The mesengenic process. Clinics Plast. Surg. 1994; 21:429-435). This process functions to continually rejuvenate various mesenchymal tissues and ensure rapid repair of tissue injuries. Such repair involves the recapitulation of the same cellular transitional events observed during embryonic development on the scaffolding of the pre-existing macro-morphology and its signaling systems. Clinical applications of these findings have been attempted (Horwitz EM, et al. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nature Medicine 5, 309-313, 1999).

MSCs have been shown to differentiate to cardiac myocytes *in vitro* under proper conditions (Makino S. Fukuda K, Miyoshi S, et al.: Cardiomyocytes can be generated from marrow stromal cells *in vitro*. J. Clin. Invest. 1999; 103:697-705). After immortalizing MSCs by prolonged culture *in vitro*, they were able to identify a single clone of adherent fibroblast like cells which, when treated with 5-azacytidine, would reproducibly differentiate into adjoining myocytes with synchronous beating. Analysis of the isoforms of contractile protein genes, such as myosin heavy chain, myosin light chain,  $\beta$ -actin, indicated that the phenotype of these cells was similar to that of fetal ventricular cardiomyocytes. After differentiation, these cells, which they called cardiomyogenic cells, acquired many morphologic features of cardiac muscle, including sarcomeres, one to three centrally located nuclei, and atrial granules. They also expressed several cardiac specific genes, including the GATA4 and Nkx2.5 transcription factors and the brain natriuretic peptide (BNP) as well as atrial natriuretic factor (ANF) genes.



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They stained positive with anti-myosin, anti-desmin, and anti-actinin antibodies. In addition, they displayed cardiac like action potentials with a shallow resting membrane potential, long action potential duration, as well 5 as a late diastolic slow depolarization current.

However, it should be emphasized that these findings were obtained and observed *in vitro* only, and no *in vivo* studies had been reported before the present invention.

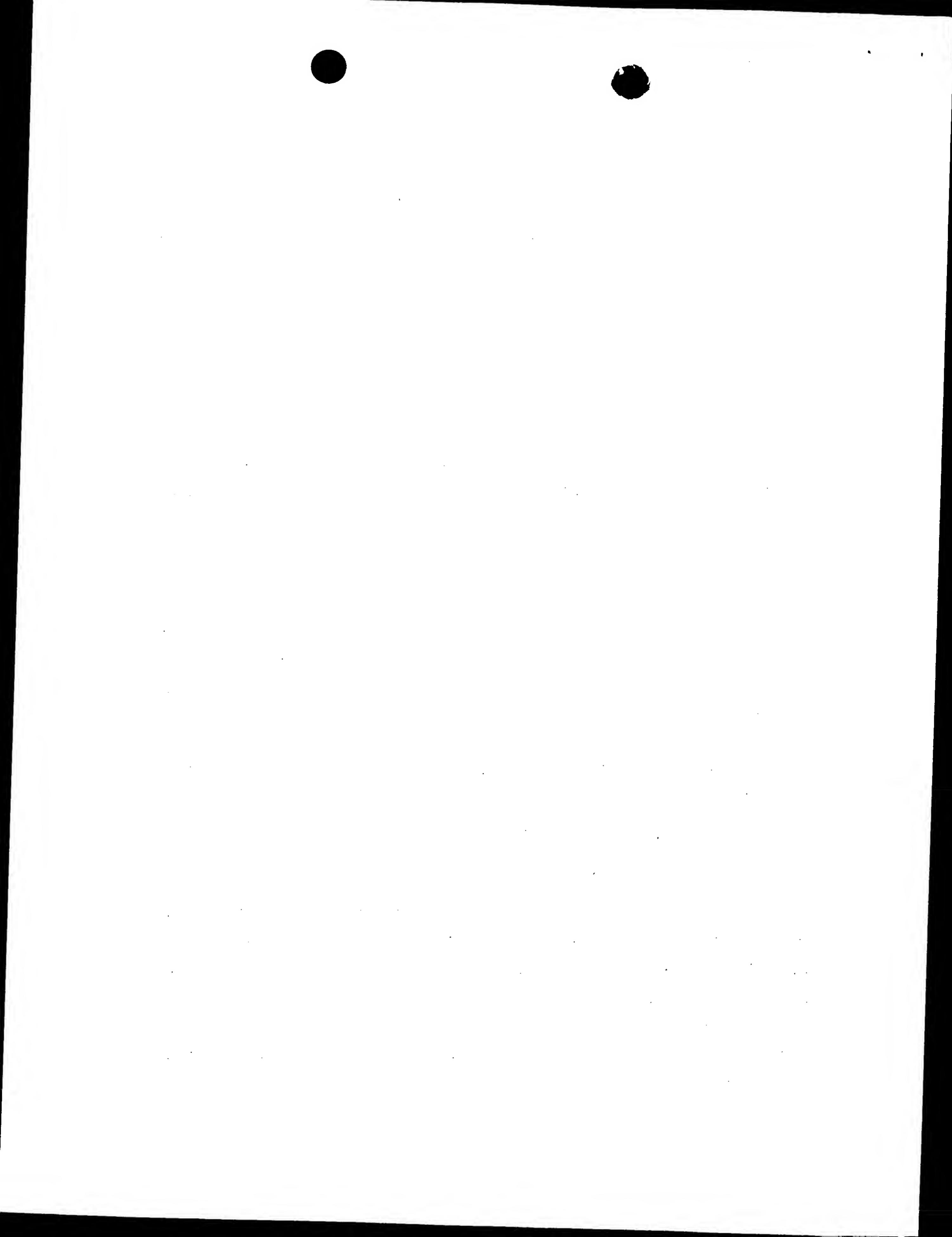
The myocardium belongs to mesenchymal tissues. In 10 accordance with an embodiment of the present invention, the myocardium was investigated as a target recipient of MSCs for effecting *in vivo* MSCs differentiation for improving cardiac function.

In accordance with an aspect of the present 15 invention, bone marrow stoma cells (MSCs) were employed in *in vivo* myocardium implantation and found to effect growth of new muscle fibers and improve overall cardiac function.

In accordance with the present invention, there is provided a use of autologous MSCs for improving cardiac 20 function wherein said MSCs are transplanted *in situ* into a myocardium.

In accordance with another aspect of the present invention, there is provided a use of autologous MSCs for improving cardiac function wherein said MSCs are 25 transplanted *in situ* into a myocardium, and differentiate into cardiomyocytes, fibroblasts and endothelial cells.

In accordance with the present invention, there is provided a method of improving cardiac function in a patient with heart failure without eliciting an immune 30 response and without sacrificing the patient's skeletal muscle; which comprises the step of transplanting autologous bone marrow stroma cells (MSCs) into said patient's myocardium to grow new muscle fibers.



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The method may further comprise the step of using a cell labeling technique to confirm survival and differentiation of implanted MSCs, and to identify said MSCs phenotype by both morphology and molecular markers.

5 The method may further comprise examining the effects of the micro-environment of implanted MSCs on their differentiation and phenotype expression.

10 The method may further comprise examining functional contribution of MSCs implanted into an ischemic segment of the myocardium.

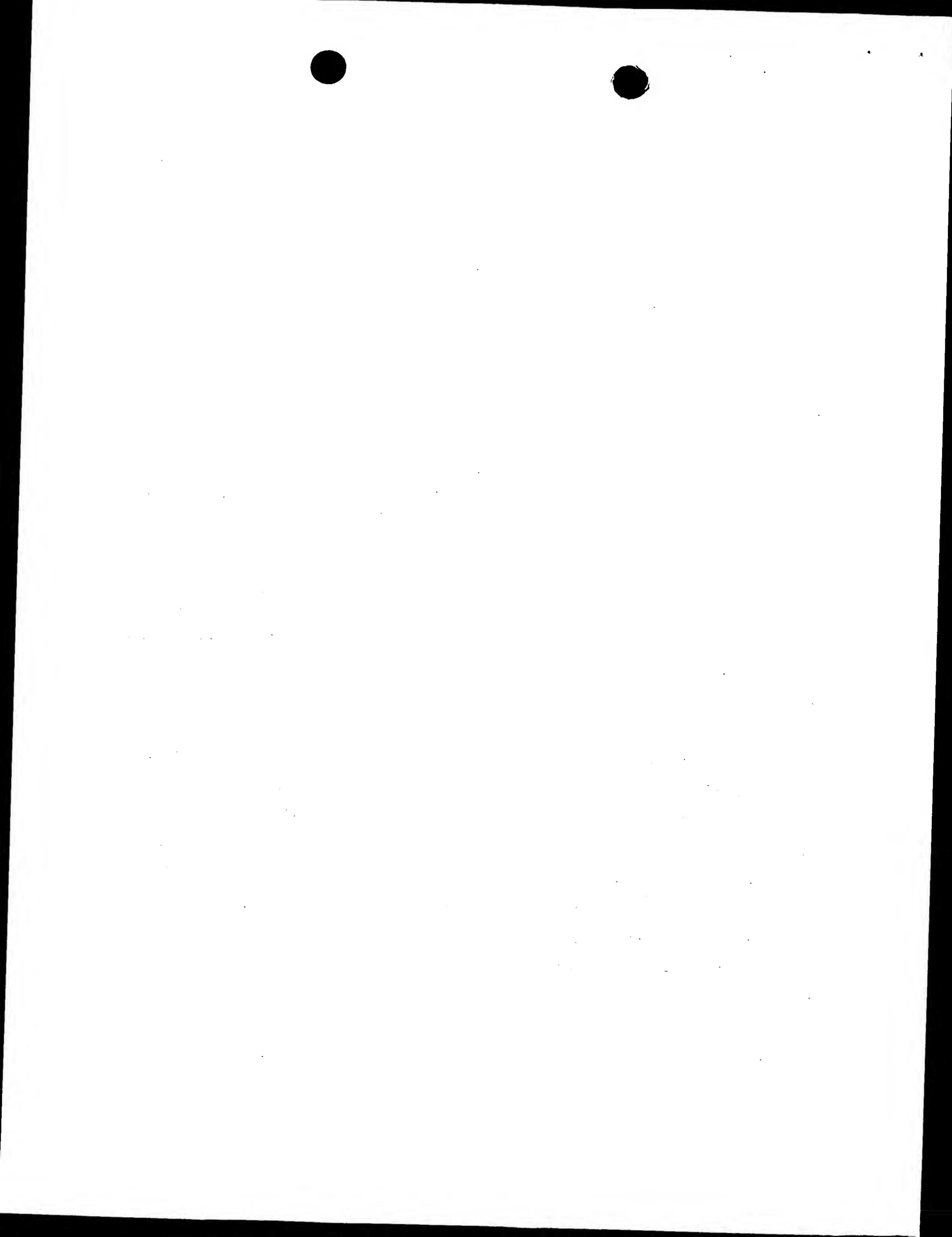
The transplanting may be effected in the myocardium *in situ*, in the myocardium artery or using a catheter from within the myocardium.

15 The transplanting may also be effected in association with angiogenesis factors.

In accordance with a further aspect of the present invention, there is provided a method of treating cardiac failure, said method comprising (a) retrieving bone marrow from a patient suffering from cardiac failure; 20 (b) isolating marrow stroma cells from said bone marrow; (c) expanding said marrow stroma cells in culture; and (d) introducing said marrow stroma cells into a myocardium of said patient.

25 In accordance with a yet further aspect of the present invention, there is provided a use of autologous marrow stroma cells for examining the effects of a myocardial micro-environment on marrow stroma cell differentiation, wherein said autologous marrow stem cells are introduced *in situ* into an ischemic segment of a 30 myocardium of an animal model.

Recently, preliminary studies were carried out using autologous bone marrow stroma cells (MSCs) as donor cells for myocardial implantation.



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In accordance with the present invention, the rationale for using MSCs as donor cells for cellular cardiomyoplasty will be evident.

##### 5 BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates four days after implantation of MSC into the myocardium; Top panel: Hematoxylin and eosin stain; Lower panel: Fluorescent microscopy picture, showing MSCs labeled with DAPI;

10 Fig. 2 illustrates four weeks post-implantation;

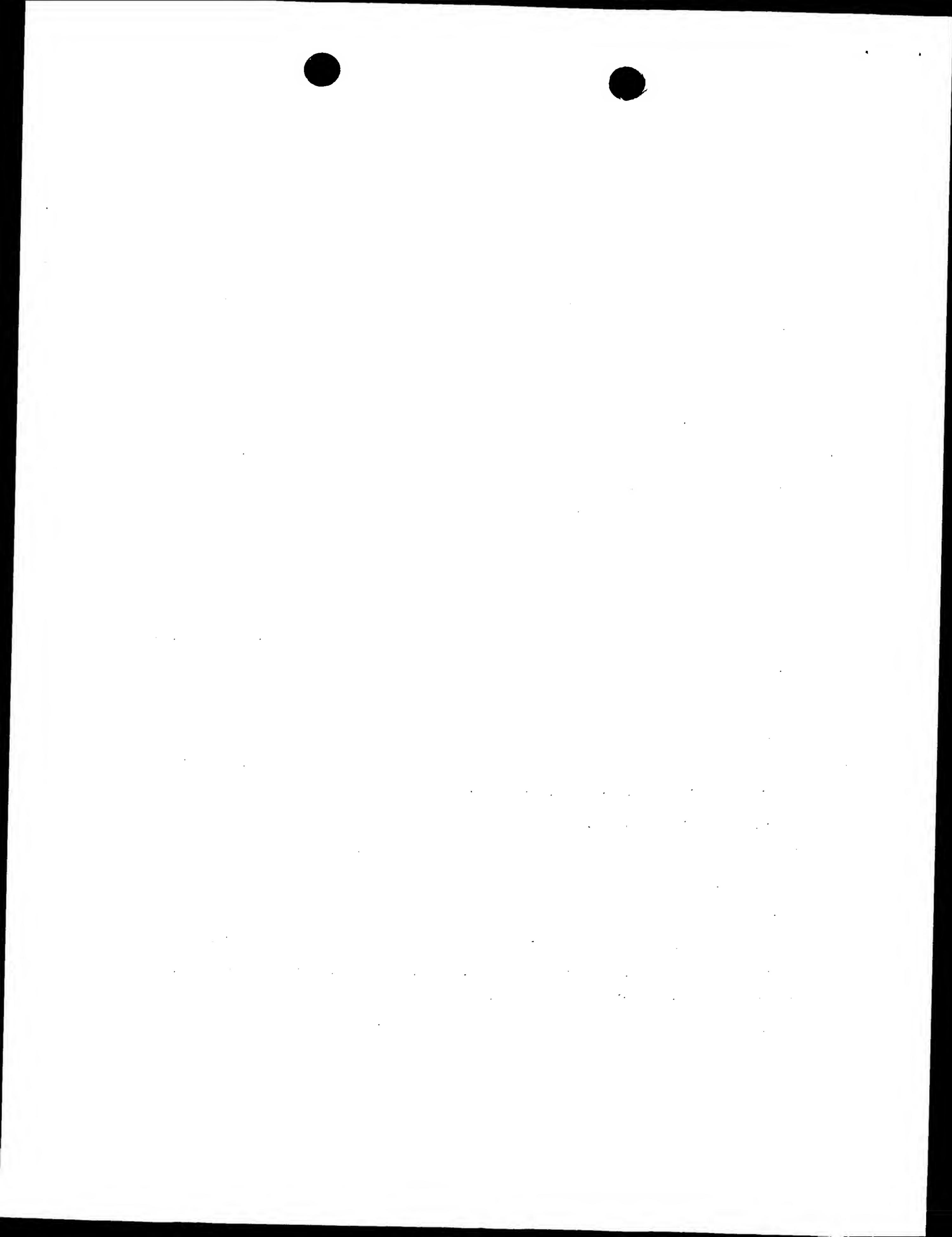
Fig. 3 illustrates specimen four weeks after MSC implantation into the myocardium; Top panel: Hematoxylin and eosin stain; Lower panel: Fluorescent cells originated from MSCs labeled with DAPI *in vitro*;

15 Fig. 4 illustrates rat MSCs morphology in culture, and in particular, a phase contrast photomicrograph of twice-passaged culture of MSCs just before implantation. Most adherent MSCs are practically fibroblastic in morphology. Scale bar represents 30 $\mu$ m;

20 Fig. 5 illustrates histochemical staining for  $\beta$ -galactosidase activity of rat MSCs in culture. The transfected MSCs showed clear staining for  $\beta$ -galactosidase activity. Transfection efficiency of the MSCs was approximately 100%. Scale bar represents 60 $\mu$ m;

25 Fig. 6 illustrates  $\beta$ -gal positive cells trapped within a coronary capillary immediately after MSCs injection. Staining for  $\beta$ -galactosidase activity followed by H & E stain. Arrow, a capillary endothelial cell. Scale bar represents 15 $\mu$ m;

30 Fig. 7 illustrates  $\beta$ -gal positive cells with morphology outside infarct scar 4 weeks after MSCs injection. Staining for  $\beta$ -galactosidase activity followed



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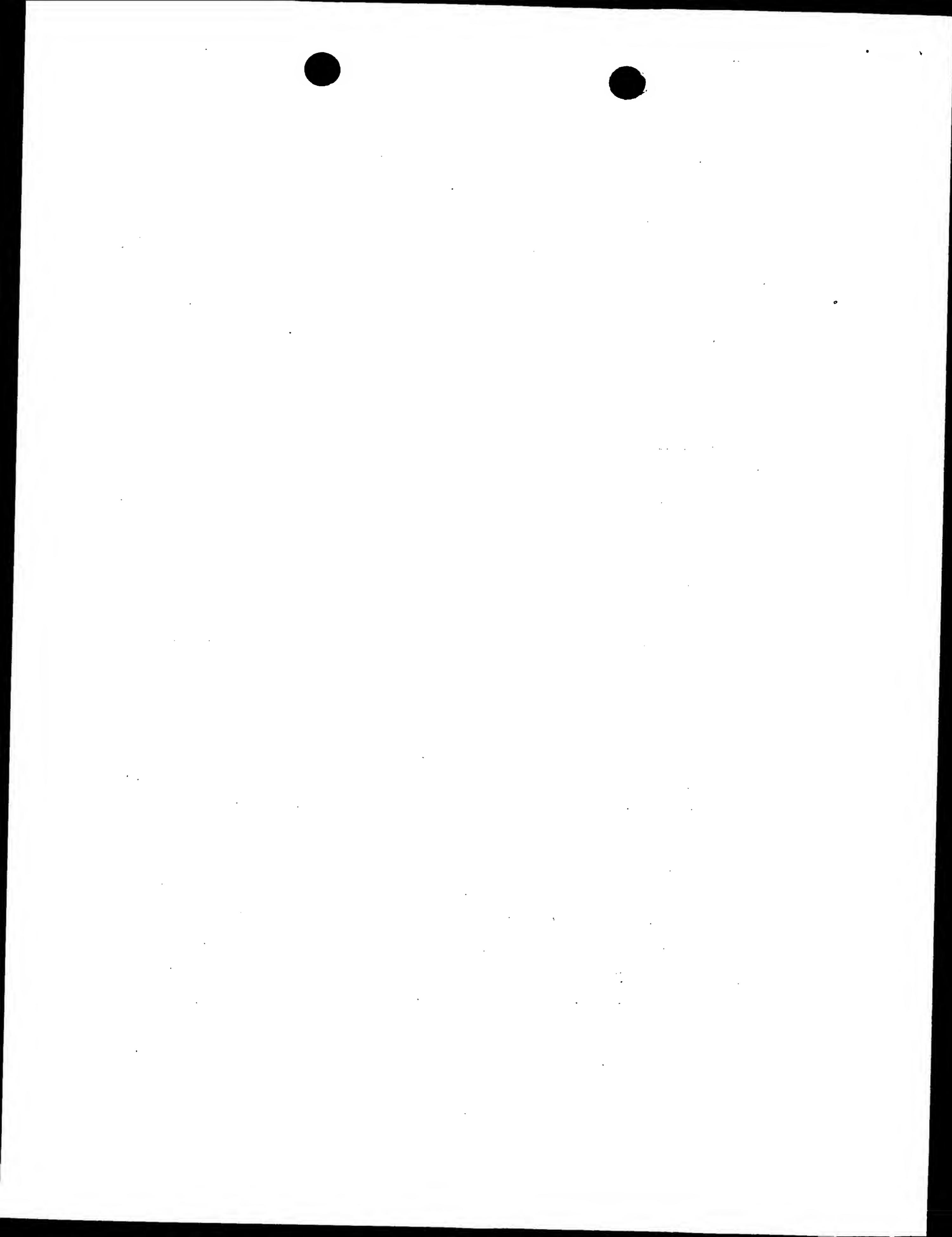
by H & E stain. Arrows, intercalated disk-like structure. Arrowhead, the nucleus of a  $\beta$ -gal positive cell. Scale bar represents 15 $\mu$ m;

Fig. 8 illustrates  $\beta$ -gal positive cells with fibroblast-like morphology in the myocardial scar 4 weeks after MSCs injection. Staining for  $\beta$ -galactosidase activity followed by H & E stain. Arrows,  $\beta$ -gal positive cells. Scale bar represents 1.5mm. (Inset) Higher magnification of the area in square. Scale bar represents 30 $\mu$ m;

Fig. 9 illustrates  $\beta$ -gal positive cells incorporated into endocardium 4 weeks after MSCs injection. Staining for  $\beta$ -galactosidase activity followed by Eosin stain. Arrow, endocardium. Scale bar represents 15 $\mu$ m;

Figs. 10A & 10B illustrate  $\beta$ -gal positive cells incorporated into coronary capillaries 4 weeks after MSCs injection. Staining for  $\beta$ -galactosidase activity followed by H & E stain. A. Outside the infarct scar. Arrow, a capillary with  $\beta$ -gal positive cells in the subendocardial fibrosis area. Arrowhead, normal myocardium. Asterisk, endoventricular space. Scale bar represents 600 $\mu$ m. (Inset) Higher magnification. Arrow, the cross section of the same capillary with  $\beta$ -gal positive cells. Scale bar represents 15 $\mu$ m. B. In the infarct scar. Arrow, the oblique section of a capillary with  $\beta$ -gal positive cells. Scale bar represents 15 $\mu$ m; and

Fig. 11 illustrates histochemical stain for connexin-43 in the intercalated discs (arrows), demonstrating the presence of gap junction unique to myocardium in the labeled (blue) myocytes.

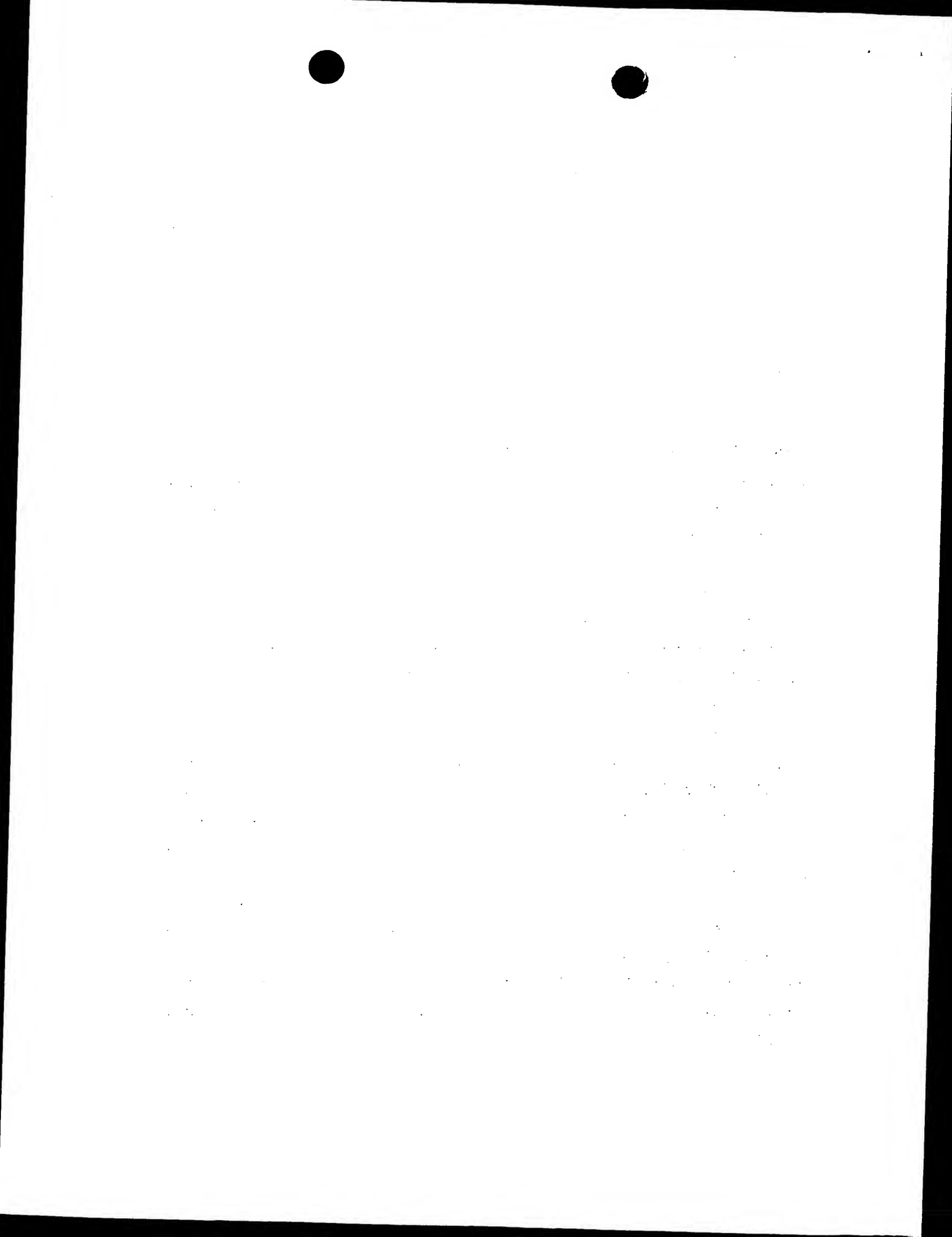


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BEST MODE(S) FOR CARRYING OUT THE INVENTION

In accordance with the present invention, there is provided means to perform myocardial implantation without eliciting an immune response and without sacrificing the 5 patient's skeletal muscle.

The ability to regenerate a functioning cardiac muscle in patients with heart failure, who have lost a significant amount of native cardiac muscle fibers through ischemic necrosis and apoptosis, has enormous therapeutic 10 potential in the treatment of heart failure. Autologous marrow stroma cells (MSCs) have been identified as target donor cells for eliciting myocardial regeneration *in vivo*. Using autologous MSCs as donor cells for cell 15 transplant therapy has a number of important advantages. In particular, by using autologous MSCs in cell transplant therapy, the need for fetal tissue and its ethical and legal controversies can be avoided, while also avoiding the need for immunosuppression. Unlike using modified cardiac 20 myocytes or established cell line myoblasts, the danger of oncogenicity can be diminished. In addition, using autologous skeletal myoblasts would require the sacrifice of a patient's skeletal muscle, which is irreplaceable. In contrast, bone marrow puncture, a routine clinical 25 procedure, can be repeated to harvest MSCs for more than one occasion. The procedure is also much less invasive, and can be easily performed in patients under local anesthesia, in contrast to the excision of a major muscle mass, such as the latissimus dorsi muscle in order to harvest satellite 30 cells. The latter procedure would likely require general anesthesia. Considering that such patients will be already suffering from severe heart failure, invasiveness of the procedure required for donor cell harvesting could be an important clinical consideration. The cell implantation

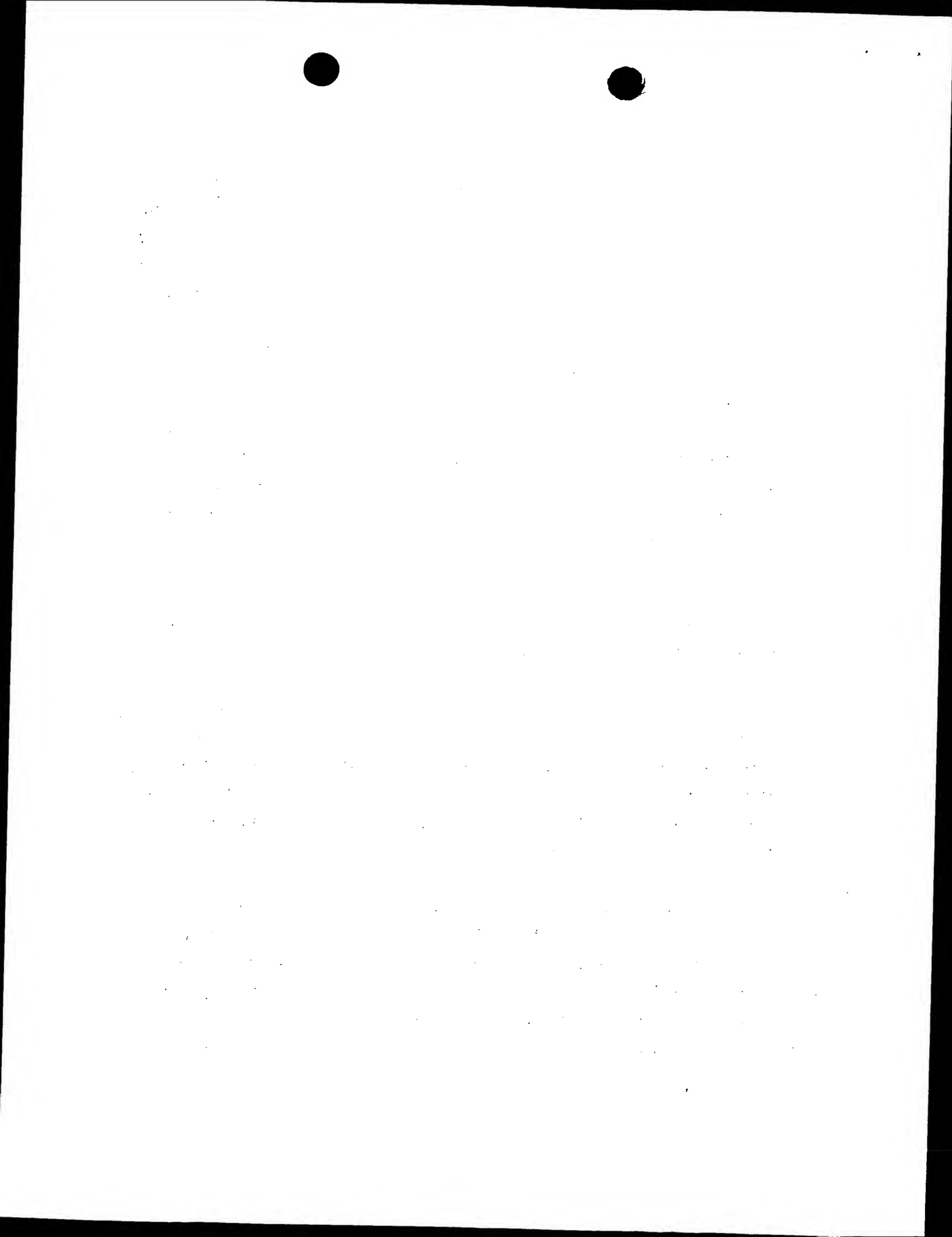


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procedure can be combined with other surgical operations, such as coronary bypass surgery, or by minimally invasive surgical techniques or by transvenous catheter injections.

Possible problems include false positive results  
5 associated with some cell labeling techniques as well as the interpretation of phenotype specificity associated with immunohistochemical findings. For example, a myosin-slow molecule may be detected both in Type I skeletal muscle fibers as well as in cardiac myocytes, and Connexin 43 may  
10 be expressed in immature myoblasts. Nevertheless, by employing several different cell labeling techniques and immunostain antibodies, correlating with histological and ultrastructural examinations, much of these uncertainties may be addressed.

15 Previous proposals to use certain sources of donor cells for cellular cardiomyoplasty are not ideal for clinical application, owing primarily to the need for fetal tissue, and/or to the need for immunosuppression (Soonpaa M.H., Koh G.Y., Klug M.G., Field L.J. Formation of nascent  
20 intercalated disks between grafted fetal cardiomyocytes and host myocardium. Science 1994;264:98-101; Li R.K., Jia Z.Q., Weisel R.D., Mickle D.A.G., Zhang J., Mohabeer M.K., et al. Cardiomyocytes transplantation improves heart function. Ann Thorac Surg 1996;62:654-61; Taylor D.A.,  
25 Atkins B.Z., Hungspreugs P., Jones T.R., Reedy M.C., Hutcheson K.A., et al. Regenerating functional myocardium: Improved performance after skeletal myoblast transplantation. Nature Medicine 1998;4:929-33; Klug M.G., Soonpaa M.H., Koh G.Y., Field L.J. Genetically selected  
30 cardiomyocytes from differentiating embryonic stem cells form stable intracardiac grafts. J. Clin. Invest. 1996; 98:216-24).

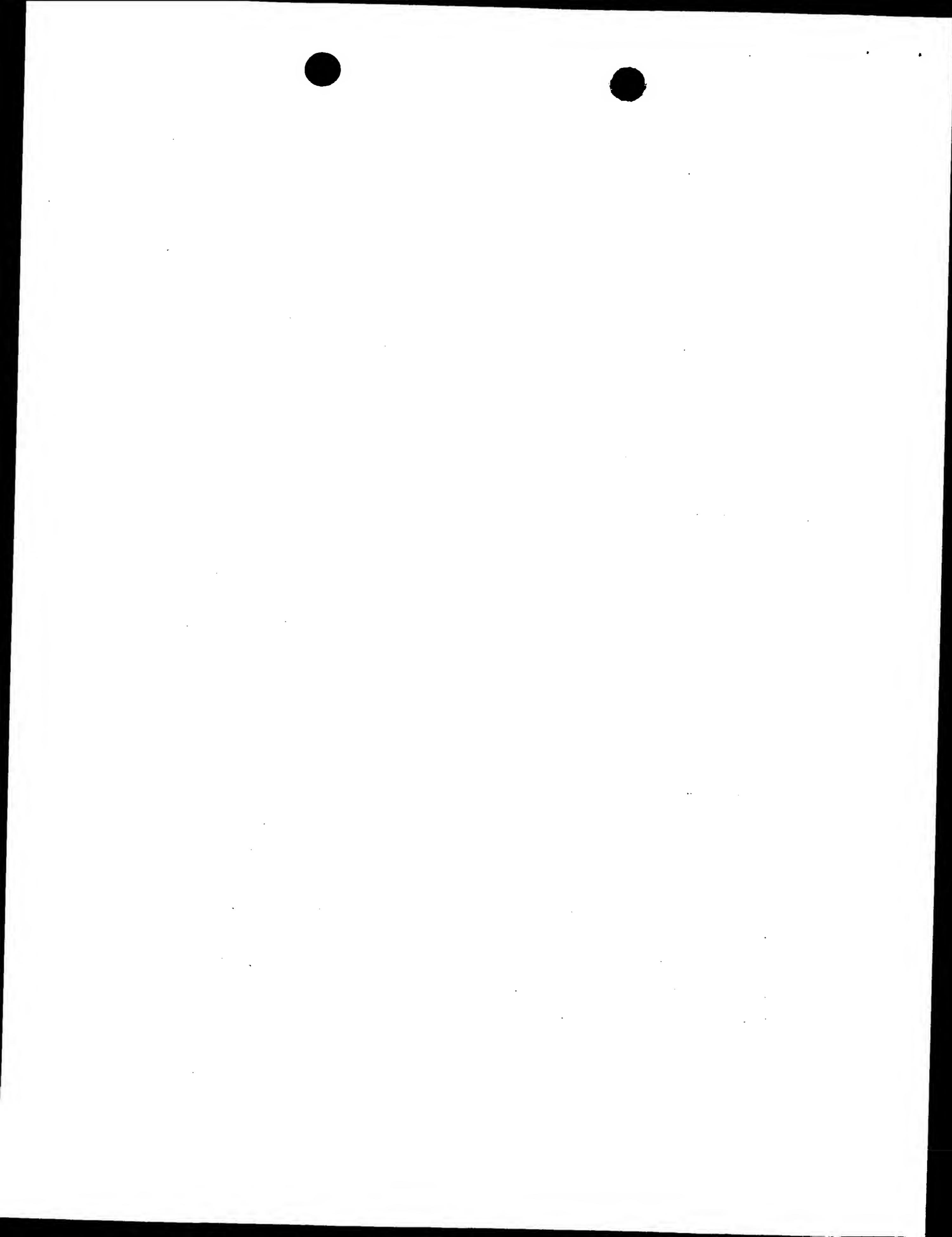


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The present invention demonstrates that the *in vivo* myocardial environment can support the growth and induce the cardiomyogenic differentiation of MSCs. Compared to other cell sources, the present invention illustrates the 5 advantages in the clinical use of MSCs for cellular cardiomyoplasty.

Grafting of cells into the myocardium requires some form of delivery system. The choice for the routes of cell implantation may depend on the pathology of the heart. Up 10 to now, most of studies in the field of cellular cardiomyoplasty were performed by direct injection of various cells into the myocardium. Though implanted cells may have the ability of migration along the ventricular surface of heart (Connold A.L., Frischknecht R, Dimitrakos 15 M, Vrbova G. The survival of embryonic cardiomyocytes transplanted into damaged host rat myocardium. J Muscle Res Cell Motil 1997;18:63-70), this procedure covers only a limited field and may require multiple injections, either through epicardium, or via the endocardium. Coronary 20 arterial delivery of donor cells to the myocardium possesses theoretical advantages, at least for certain types of heart failures. Thus, in order to optimize the strategy for cell implantation, the feasibility of delivering MDSCs by selective infusion into the coronary 25 circulation was evaluated.

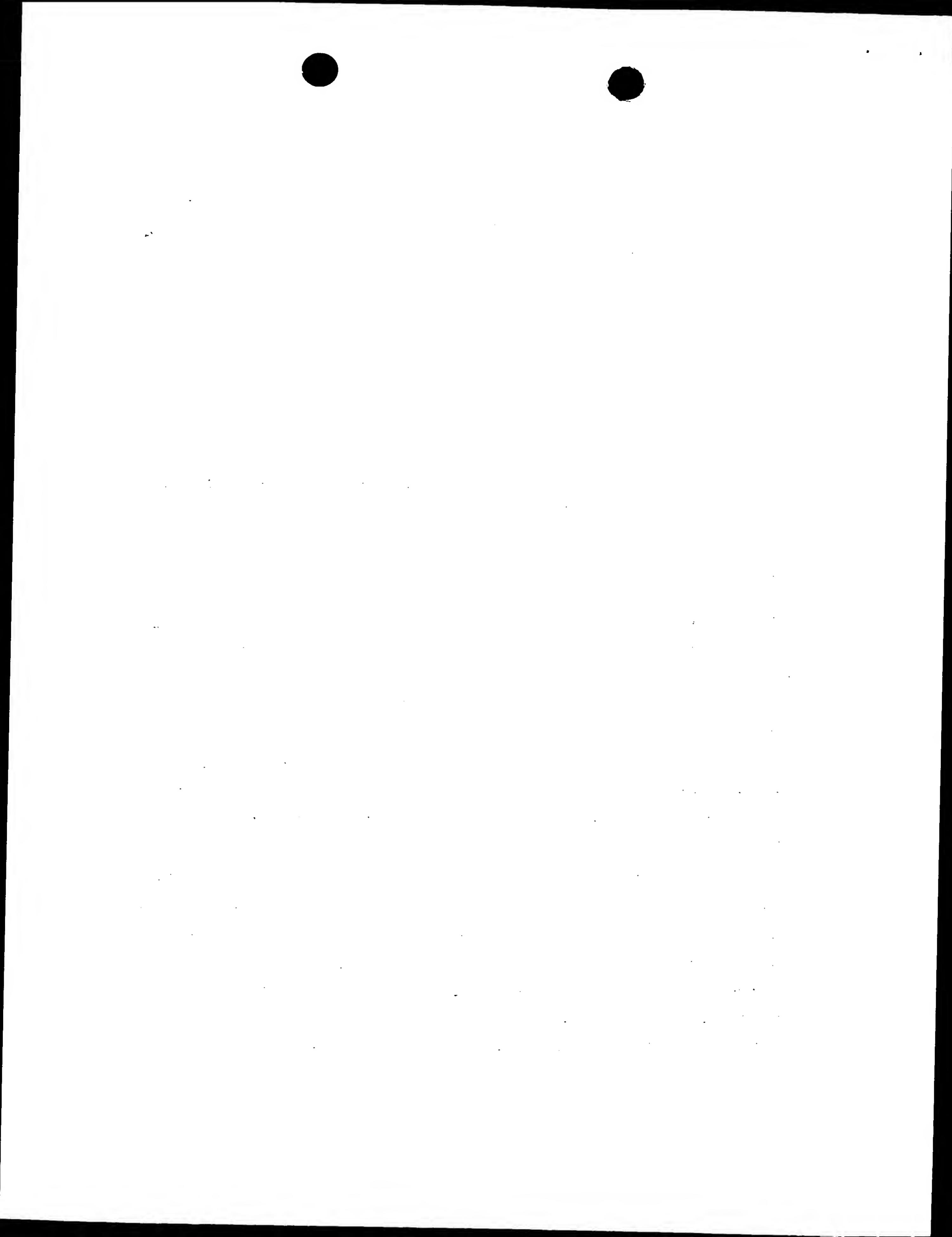
In accordance with the present invention MSCs are infused into coronary artery and appear to repopulate the heart. Further, signals originating in the cardiac milieu appear to modify the developmental program of the infused 30 MSCs. Studies conducted in accordance with the present invention further confirm the residence of MSCs outside of the capillary bed, and illustrate the structural



interactions between the host myocardial tissues and the implanted MSCs.

To trace the fate of infused MSC according to an embodiment of the present invention, MCSs were retrovirally transfected with  $\beta$ -gal reporter gene for cell labeling. Compared with other virus-based gene transfer, retrovirus has less immunological response and longer gene expression (Onifer SM, White LA, Whittemore SR, Holets VR. *In vitro* labeling strategies for identifying primary neural tissue and a neuronal cell line after transplantation in the CNS. *Cell Transplantation* 1993;2:131-149). Using current transfection model, the transfection efficiency in culture is approximately 100% without obvious adverse effect on the cell growth. Our *in vivo* control studies, including infusion of MSCs culture supernatant, non-transfected MSCs or lysed transfected MSCs while performing the same read-out, confirmed the specificity of this cell labeling technique.

Immediately after the infusion, the MSCs were trapped within the coronary capillaries in the non-infarct area. The reason MSCs could not be found in the infarct scar at this time may be related to the complete occlusion of involved coronary artery (Left coronary artery). However, 4 weeks after infusion, the MSCs could be found both in the infarcted scar and in non-infarct area outside the vascular structure. The mechanism of the translocation of MSCs from the vascular lumen into the myocardial interstitium is unknown. MSCs may migrate out of the vasculature and move from the non-infarct area to the infarction scar. The other possible explanation is that scar of myocardial infarction is not a completely dead tissue. The reason why MSCs can only be found in 60% of rats (6 out of 10) 4 weeks after injection may be related



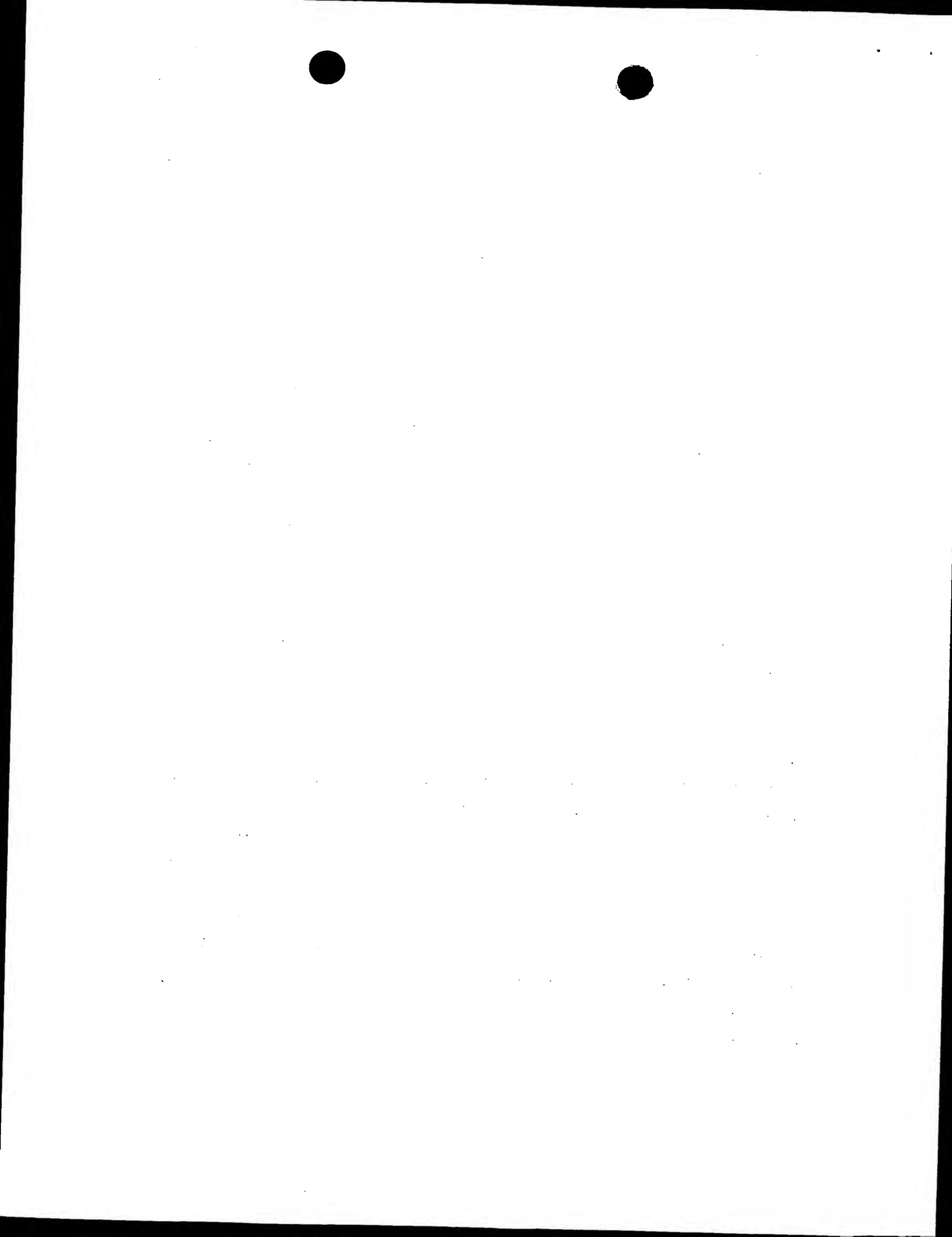
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to the infusion technique. By using this model of briefly clamping the ascending aorta distally, some cells may have leaked out of the puncture hole on the ascending aorta. Others may escape through the blood stream to distal organs  
5 and tissues.

MSCs in different myocardial microenvironments clearly have different fates. In the non-infarct area, they express the phenotypes of normal cardiomyocytes and connected with surrounding host cardiomyocytes by  
10 intercalated disk-like structure. In the infarct scar, they appear primarily fibroblast-like. This homing ability and the capability to acquire the phenotypes of different target tissues suggest that the microenvironment plays a significant role for the differentiation of these cells.

15 According to the present invention, in contrast to myocardial scar tissue, the normal myocardial microenvironment appears to enable newly arrived cells to be exposed, in an appropriate sequential manner, to various  
20 cardiomyogenic specific growth factors and differentiation molecules, such that the infused MSCs could develop into fully mature cardiomyocytes. The fibroblast-like MSCs seen in the infarction scar could have differentiated into primary fibroblast, which are mature mesenchymal cells or they could still maintain the multipotent differentiation  
25 ability for future maturation.

The studies of the present invention revealed that some  $\beta$ -gal positive donor cells differentiate into endothelium, which was incorporated into capillaries in the infarct and non-infarct areas. Accordingly, these marrow-derived endothelial progenitor cells are likely to be involved in the angiogenesis and vasculogenesis in the remodeling process of myocardial infarction. Thus furhter  
30



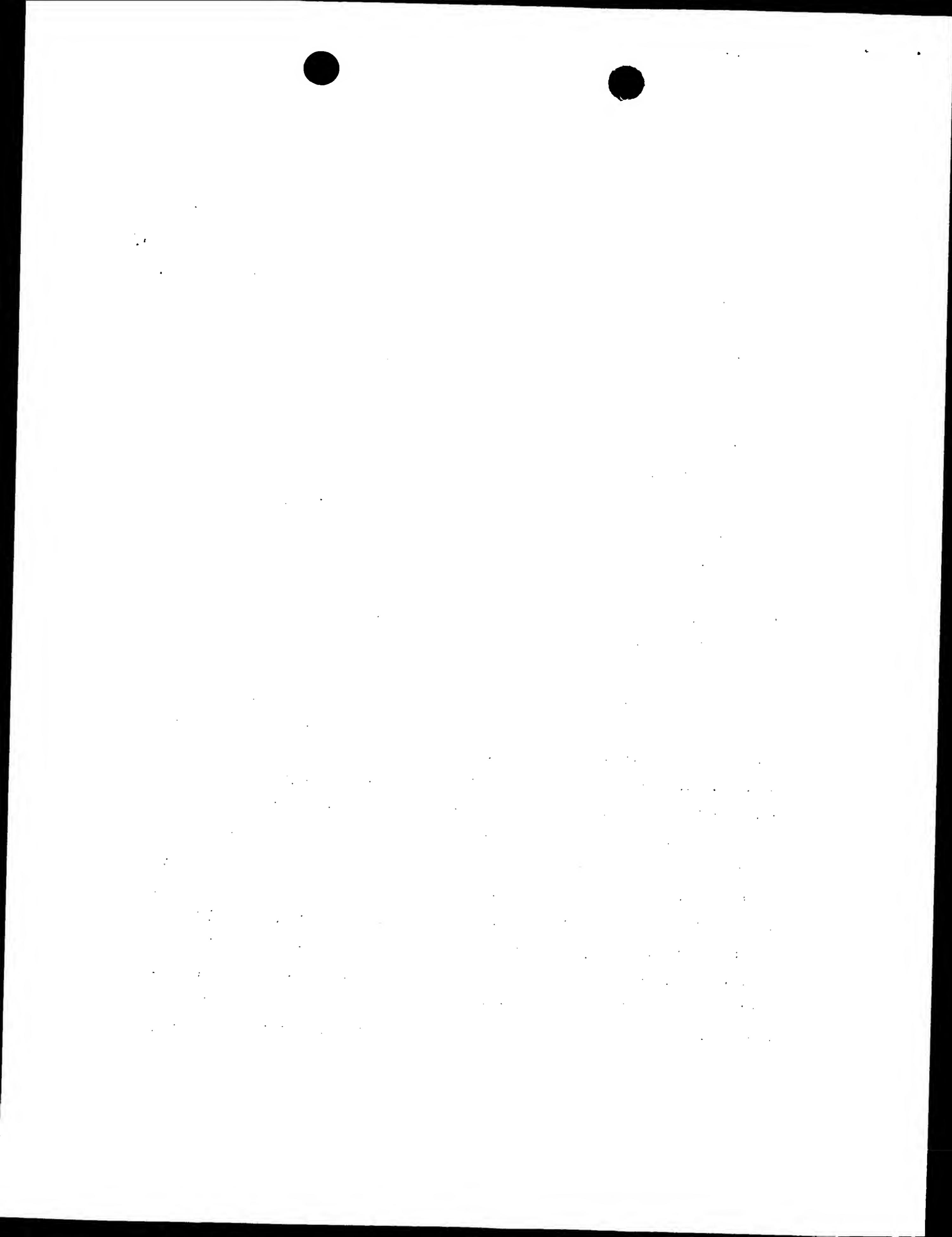
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suggesting the potential of use of MSCs for implantation into the myocardium to improve heart function.

In addition, it has been shown that some cells in MSCs culture are positive for Factor VIII-associated antigen (Singer JW, Charbond P, Keating A, Nemunaitis J, Raugi G, Wight TN, et al. Simian virus-40 transformed adherent cells from human long-term marrow cultures: Clone cells produced with "stromal" and hematopoietic characteristics. Blood 1987;70:464-474), suggesting an endothelial origin. Shi et.al suggested that a subset of cells localized in the bone marrow could be mobilized to the peripheral circulation and colonize endothelial flow surfaces of vascular prostheses (Shi BQ, Rafii S, Wu MHD, Wijelath ES, Yu C, Ishida A, et al. Evidence for circulating bone marrow-derived endothelial cells. Blood 1998;92:362-367).

Both localized site-specific and global delivery of autologous MSCs may be of potential therapeutic benefit in view of different cardiac pathology. For example, intra-coronary delivery of MSCs may be more suitable for the treatment of heart failure due to diffuse cardiomyopathy. Furthermore, the present invention demonstrates that when expanded marrow-derived stromal cells are delivered to the coronary circulation of an infarcted heart, they are capable of populating the heart and differentiating along several lineages including cardiomyocytes, fibroblast and endothelial cells.

The findings of the present invention suggest that infarcted heart muscle can signal mobilization of MSCs to enter circulation, and reach the coronary artery, where they may participate in myocyte replenishment, reactive fibrosis and scar formation as well as angiogenesis in the post-infarct pathophysiological remodeling process,



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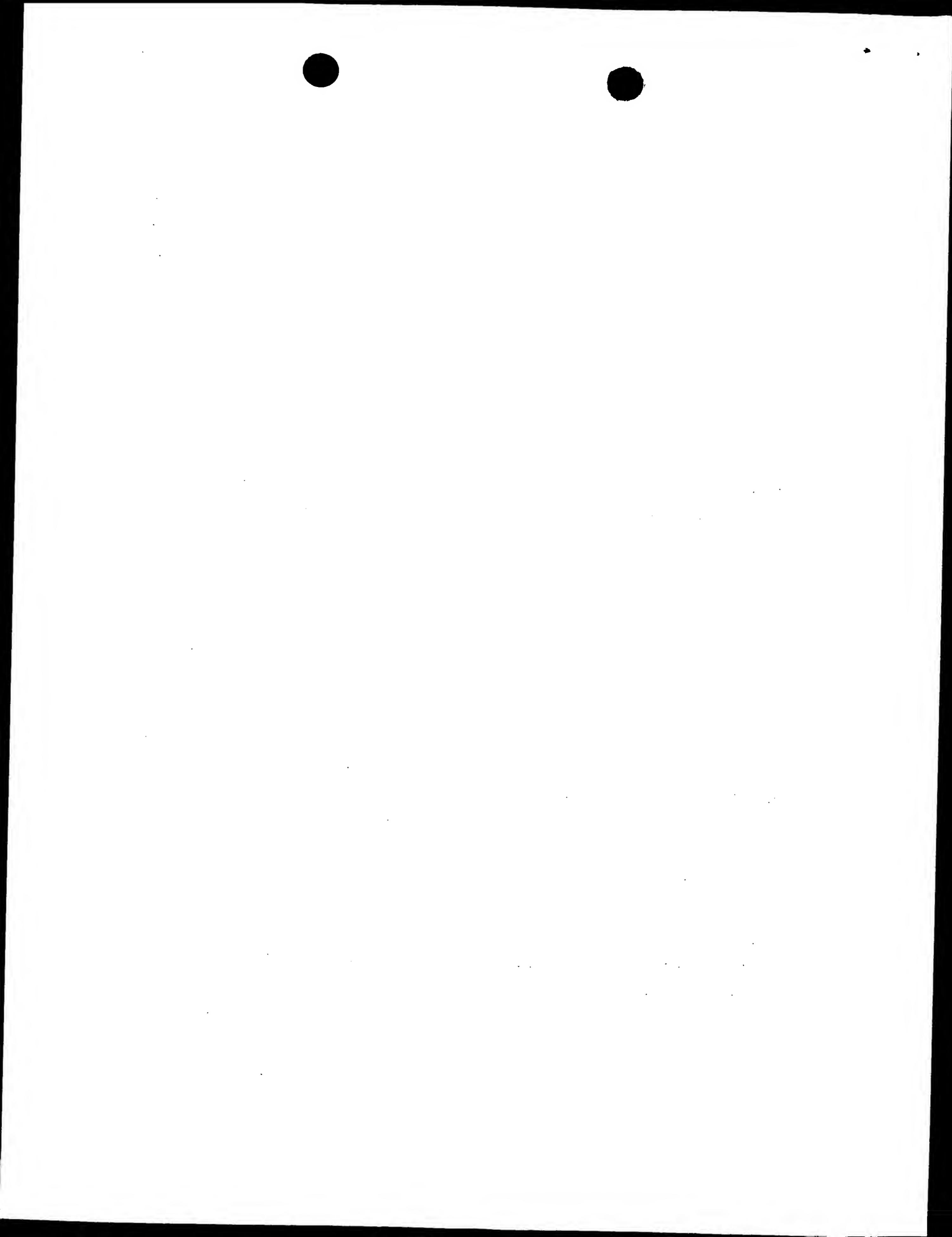
involving both the infarcted segment and the remote non-infarcted areas. Additional studies in accordance with the present invention will further elucidate the role of MSCs in myocardial infarction and the clinical applications of 5 MSCs implantation. It is fully contemplated that the findings presented in accordance with the present invention will enable therapeutic modulation of the remodeling process after myocardial infarction, in both animals and humans.

10

#### Example I

##### Preliminary Studies

In our preliminary study, isogenic rats were used as donors and recipients, since as in the autotransplants, 15 this model obviates the need for immunosuppression. Lewis rats weighting 175 to 200 grams were used in all experiments. As will be described in detail below, femoral and tibial bones were explanted from the donor rats and used as the source for bone marrow stroma cells. Details of 20 isolation, plating and passaging techniques for MSC will also be described below. The MSCs were expanded in culture for 2 to 3 weeks and labeled with DAPI (4', 6-diamidino-2-phenylindole) before transplanting them into the lateral wall of the left ventricle of the recipient rat hearts. 25 This was accomplished by direct injection of MSC suspension using a 28-gauge needle. At different time intervals ranging from 2 days to 4 weeks following the implantation, the hearts were harvested and studied histologically. The hearts at the implant sites were sectioned serially, and 30 stained with hematoxylin and eosin. Slides of the adjacent section to the above were examined under fluorescent microscopy to identify DAPI, which upon binding to the DNA



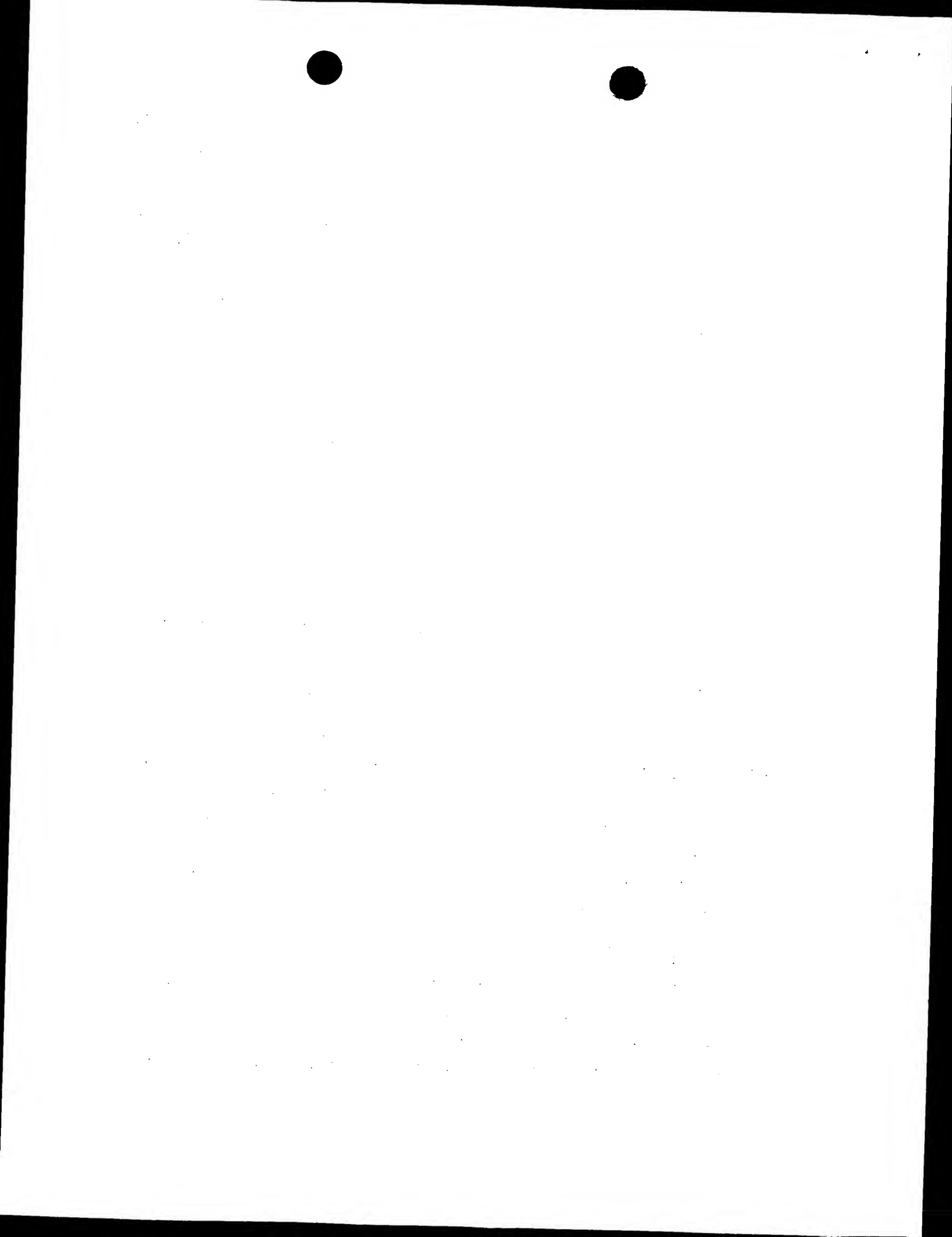
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of the MSCs prior to implantation, can be recognized as fluorescent positive cells.

The sections (6  $\mu\text{m}$ /thick) were also examined immunohistochemically using antibodies against myosin-slow molecules. Other serial sections from these specimens are currently undergoing studies using antibodies against the cardiac gap junctional protein, Connexin-43, and other phenotype specific antibodies, for immunolabeling and staining.

Fig. 1 illustrates a microphotograph obtained 4 days after implantation. The implant site shows a needle track created during the process of injecting the MSCs, with some inflammatory response and fibrosis within the needle track. Fluorescent microscopic examination demonstrated the presence of the labeled MSCs implanted. Fig. 2 shows labeled cells in the injection site, immunolabeled with myosin-slow antibodies which stains red; DAPI labeled fluorescent cells within needle track made during implantation; and immunohistochemical stain with antibody against slow myosin heavy chain, which shows red color. The triangle marker points to the native cardiac myocyte, and arrows point to the appearance of myosin molecules in the cytoplasm of implanted cells.

The deep red color shows myosin-slow heavy chains in the native muscle (triangle), whereas the red stain adjacent to the labeled cells suggest the synthesis of myosin-slow molecules in the implanted cells (arrows). Fig. 3 is a photograph taken a short distance away from the implant needle track, showing migrated or infiltrated MSCs appearing to have differentiated fully and were incorporated into cardiac muscle fibers, morphologically indistinguishable with the native myocardium. Clear labeling of these cells can be demonstrated under



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fluorescent microscopy. Morphologically, they appear identical to the native myocardial fibers. Photographs were taken from the myocardium adjacent to the needle track, where the implanted cells had migrated or infiltrated.

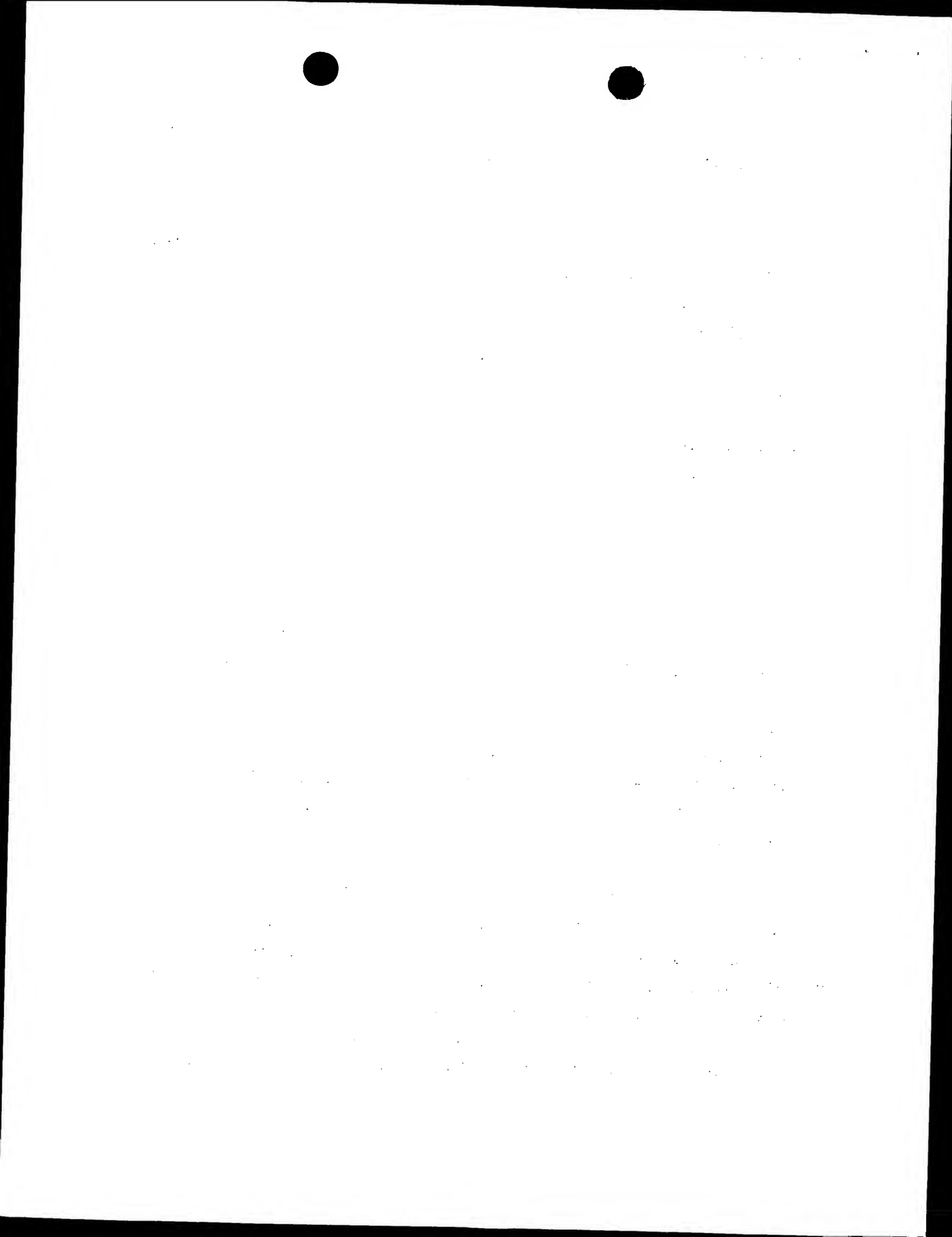
5        Additional studies are described below to confirm these findings, as well as to further elucidate the phenotypes of the new muscle using additional specific antibodies. These preliminary studies, however, clearly demonstrated various experimental techniques, ranging from  
10      isolation, culture and identification of MSCs, as well as implanting of these cells to the rat hearts with virtually no mortality.

15      Experimental Protocol: The Rationale for Experimental Models

In clinical application of the present invention, use of autologous MSCs for cardiac implantation to improve cardiac function is particularly advantageous in that it avoids the need for immunosuppression. Relatively  
20      inexpensive isogenic Lewis rats were chosen for the preliminary study. The preliminary investigation described above indicates that this is a useful and reliable model, with little operative mortality for cardiac cell implantation resulting. Furthermore, the capability to  
25      perform coronary artery ligation and sequential echocardiographic studies in such animals is confirmed and the feasibility of proposed experiments in accordance with the present invention is supported.

30      Experimental Design

For each rat receiving cardiac cell implant (experimental animal), there will be an isogenic rat to serve as the donor of bone marrow stroma cells. Another group of sham operated rats (controls) will undergo



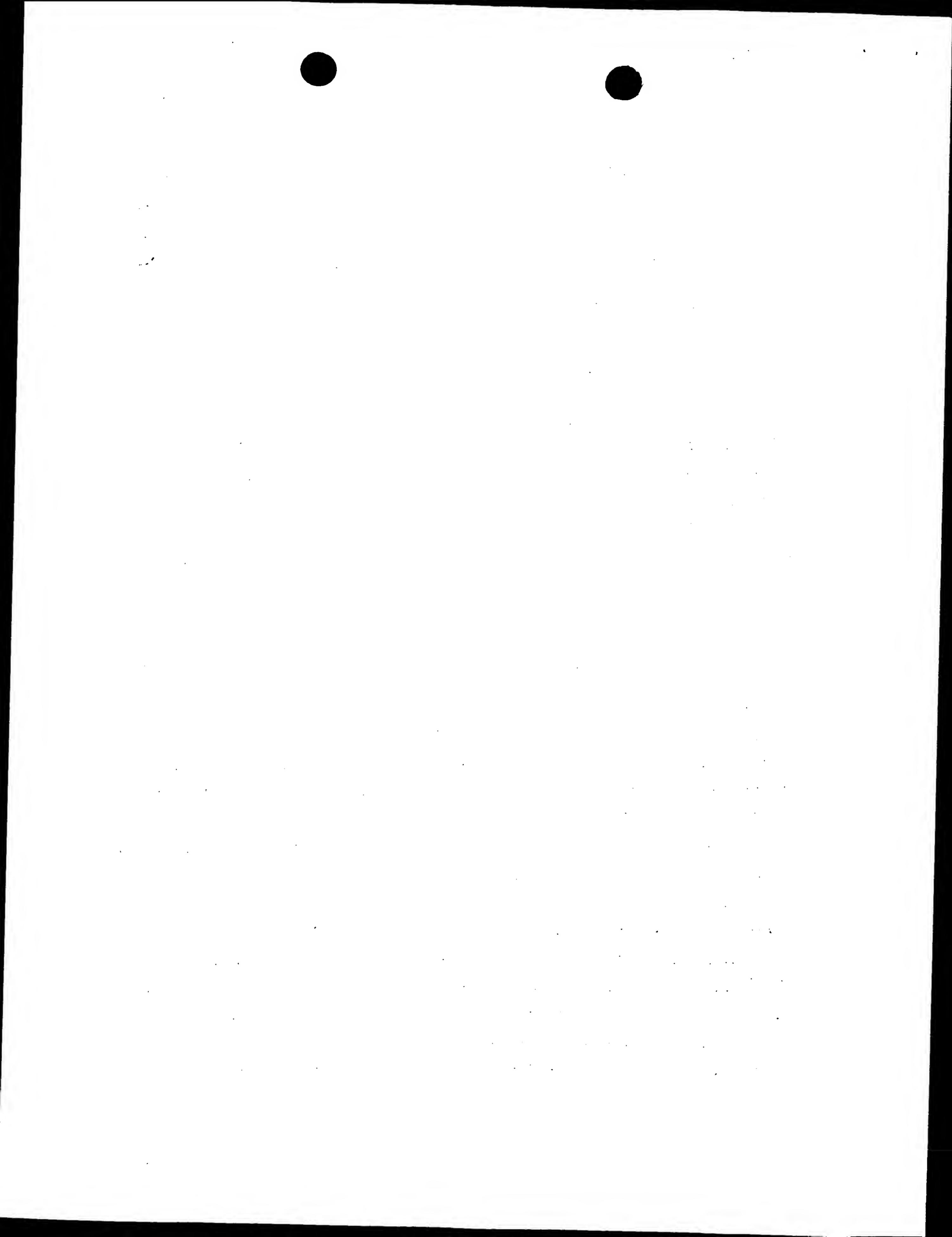
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identical surgical procedures as the experimental animals, but will receive injection of cell culture media without MSCs.

a) Donor rats will be sacrificed, and their femoral 5 and tibial bones will be used to isolate, select and culture MSCs *in vitro* for 2 weeks using the technique described below. Then the cells will be collected, labeled and injected into the myocardium of the experimental recipient rats.

b) Cell implant recipient (experimental) rats: The prospective future recipients for cell implant will undergo thoracotomy and ligation of the anterior descending coronary artery (see below). The chest will be closed and the animals monitored with weekly echocardiographic studies 15 to observe changes in the ventricular wall motion of the ischemic zone for 2 weeks. In the second thoracotomy, these rats will receive injection of isogenic MSCs cultured and labeled *in vitro*. The injections will be made through a 26-gauge needle into the anterolateral wall of the left 20 ventricle, both at the center of the infarct zone, as well as at the peripheral border zone between the infarcted and non-infarcted cardiac muscles. Following the implant procedure, the chest will be closed and studied weekly using echocardiography as described below. They will be 25 sacrificed after cell implantation at an interval of 4 days, 2 weeks, 4 weeks, and 3 months, with a sample size of 10 rats each. Frozen sections will be made serially through the implant site, at a thickness of approximately 6  $\mu\text{m}$  each, and slides will be processed for fluorescent microscopic, 30 histological, immunohistochemical and electron microscopic studies as described in detail below.

c) Sham operated (control) rats: This group will undergo coronary artery ligation and cardiac implantation



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procedures exactly as described above for the experimental group. However, instead of receiving cultured and labeled MSCs, they will receive the same volume of culture media only. They will be harvested at the same time intervals and  
5 studied in the same manner as described for the experimental animals.

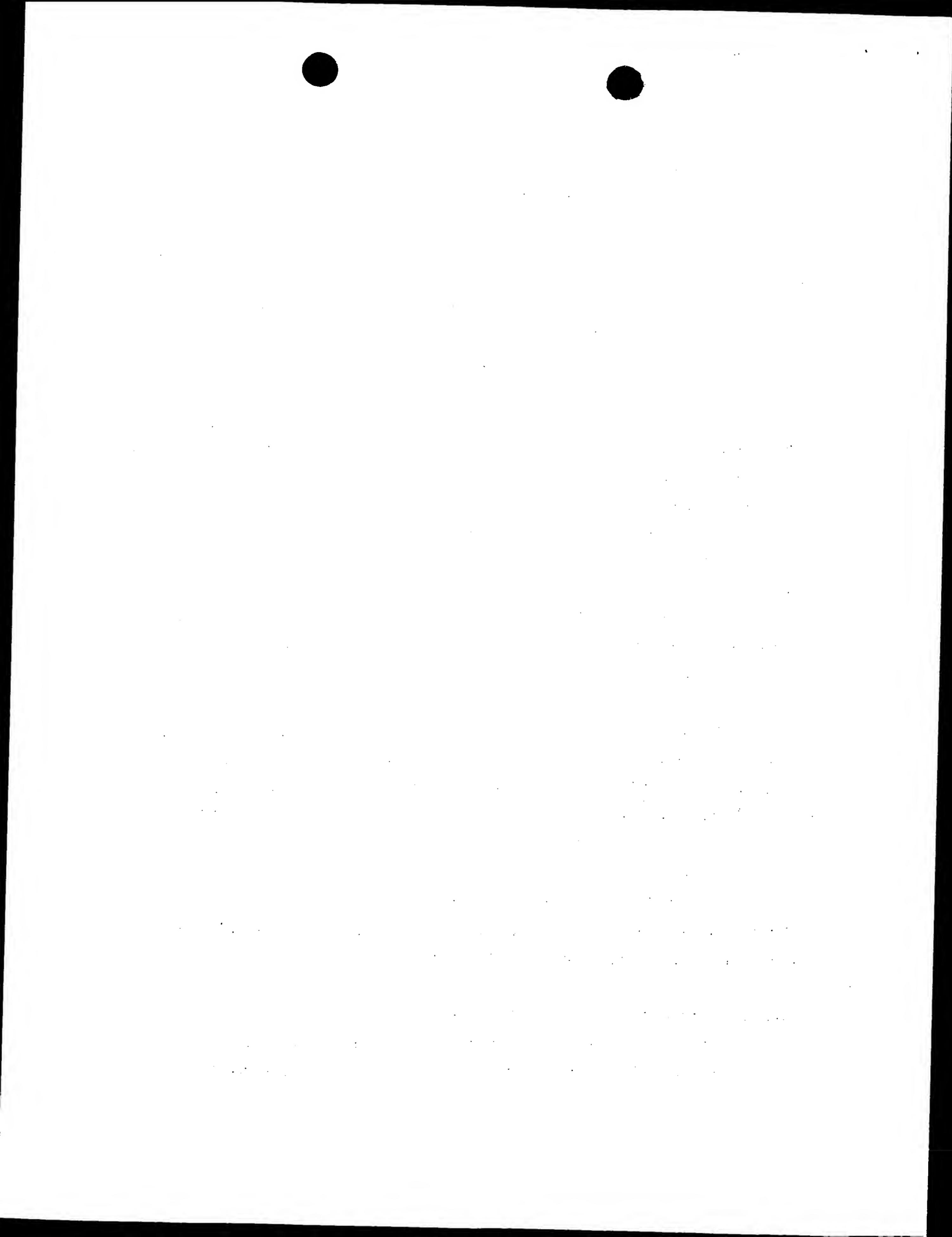
#### Sample Size

The sample size of 10 was based on our preliminary  
10 study as this is a highly reliable model that resulted in minimal operative mortality. In the future, wall motion studies will be performed to calculate the sample size required.

All animals will receive humane care and all  
15 experiments will be performed according to the "Guidelines to the Care and Use of Experimental Animals" of the Canadian Council on Animal Care.

#### 20 Isolation, Plating and Passaging Techniques of Bone Marrow Stroma Cells

Isolation and primary culture of MSCs will be performed according to Caplan's method. After overdose with pentobarbital (100 mg/kg given intraperitoneally) (MTC Pharmaceuticals, Cambridge, Ontario), the femoral and  
25 tibial bones of the donor Lewis rats (weighing 175 to 200 grams) will be collected and the adherent soft tissue removed. Meticulous dissection of the long bones will be carried out in order to remove soft tissue to ensure that myogenic precursors are not carried into the bone marrow preparation. Both ends of the bones will be cut away from  
30 the diaphysis with bone scissors. The bone marrow plugs will be hydrostatically expelled from the bones by insertion of 18-gauge needles fastened to 10 ml syringes filled with complete medium; the needles are inserted into



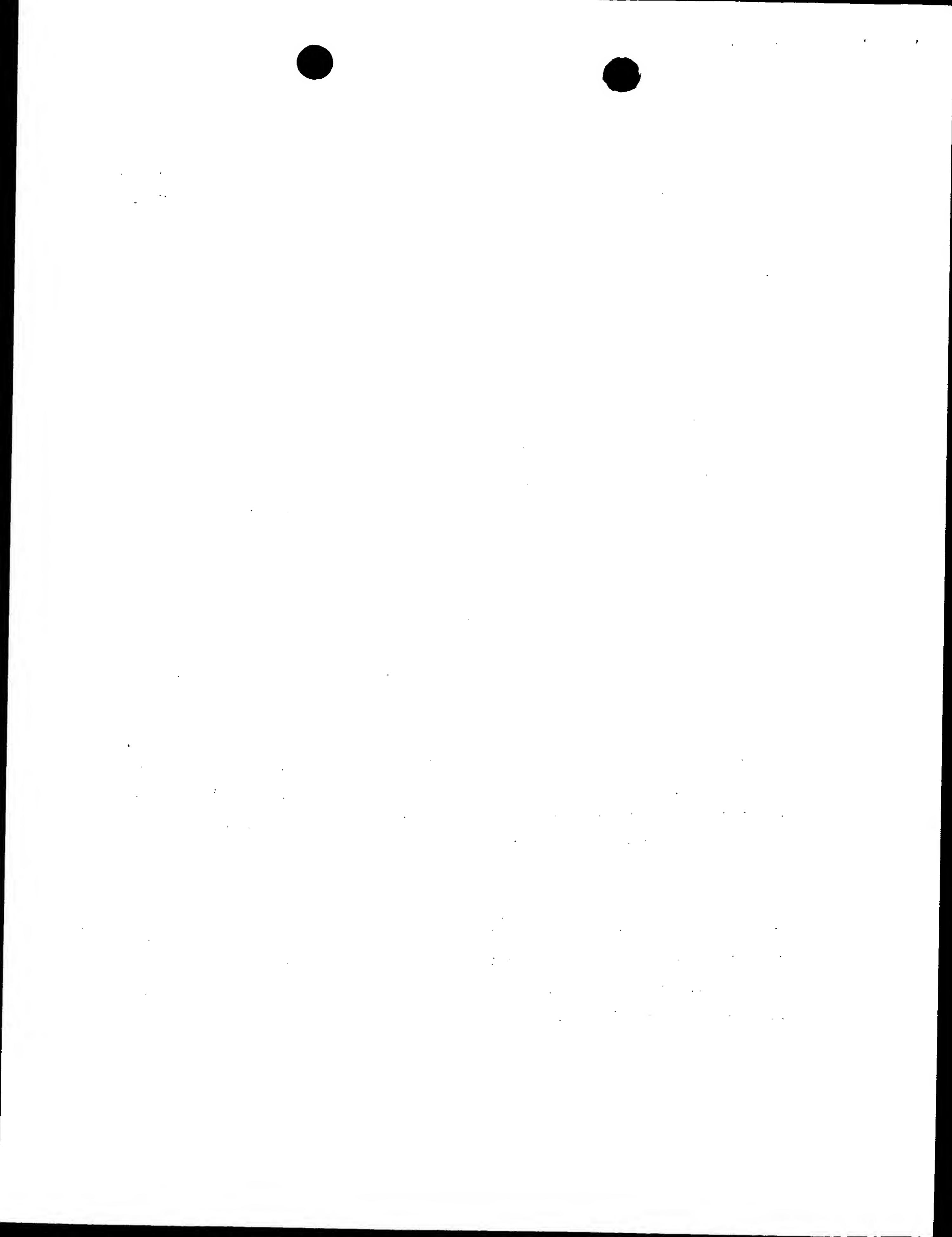
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the distal ends of the femoral and proximal ends of the tibial bones, and the marrow plugs expelled from the opposite ends. The marrow plugs are disaggregated by sequential passage through 18-gauge, 20-gauge and 22-gauge 5 needles and these dispersed cells are centrifuged and resuspended twice in complete medium. Cell viability is assessed by the trypan blue exclusion test. After the cells are counted in a hemocytometer,  $5 \times 10^7$  cells in 7-10 ml of complete medium are to be introduced into 60 mm polystyrene 10 tissue culture dishes (Corning, Inc., Corning, NY), which are coated in advance with a layer of laminin (Sigma) to promote marrow stroma cell adherence. Three days later, the medium is changed and the non-adherent cells discarded. The medium is completely replaced every 3 days. In 15 approximately 10 days after seeding, the dishes will become nearly confluent and the adherent cells can be released from the dishes with 0.25% trypsin in 1 mmol/L sodium ethylenediaminetetraacetic acid (Gibco Laboratories, Grand Island, NY), split 1:2, and seeded onto fresh plates. After 20 these twice passaged cells become nearly confluent, they can be harvested and used for implantation experiments described below after being labeled with DAPI. The "complete medium" mentioned above for our culture consists of Dulbecco's modified Eagle's medium (DMEM, Gibco 25 Laboratories) containing selected lots of 10% fetal calf serum (FCS; JR Scientific Inc., Woodland, CA), and antibiotics (Gibco Laboratories; penicillin G, 100 U/ml; streptomycin 100  $\mu$ g/ml, amphotericin B 0.25  $\mu$ g/ml) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

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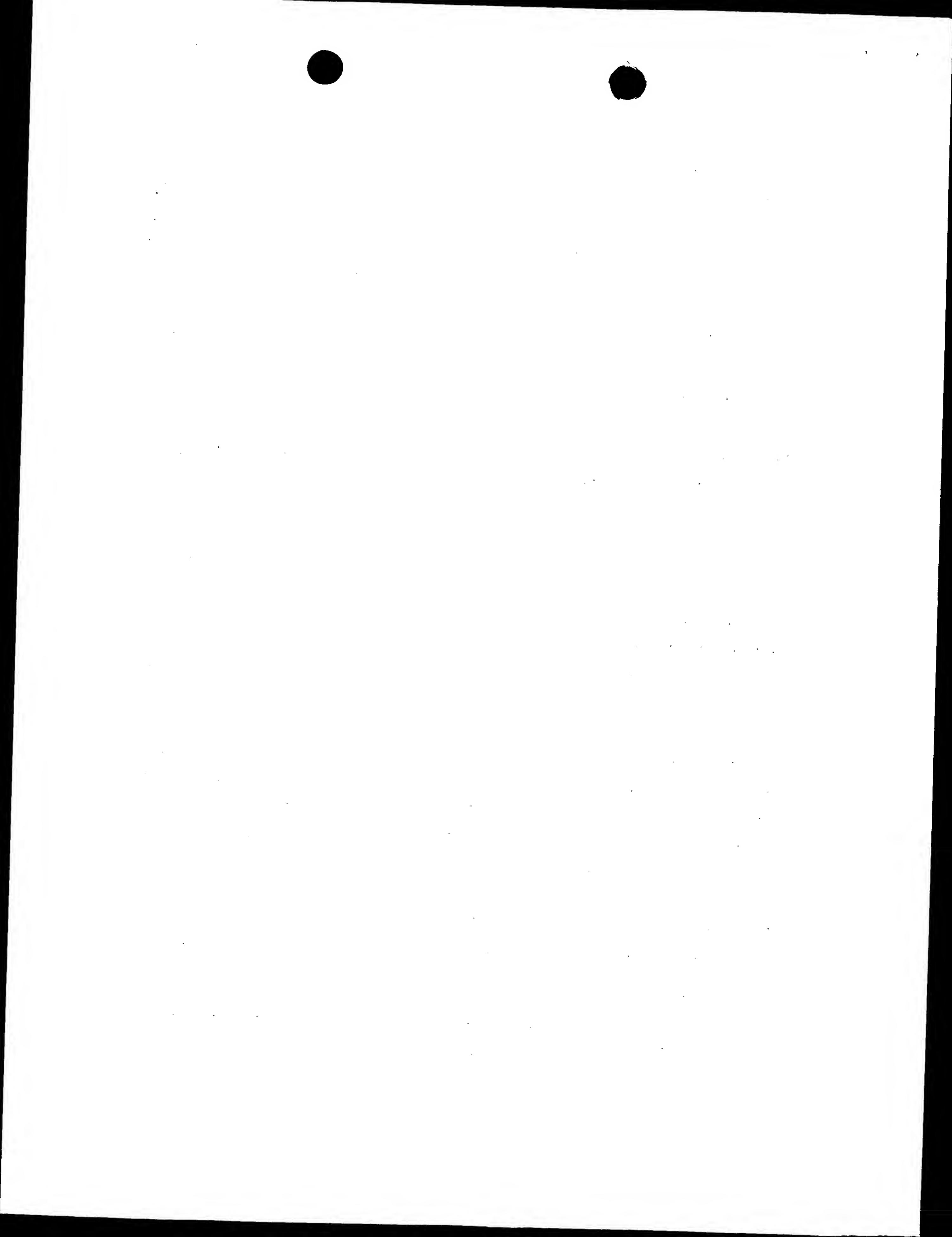
#### Bone Marrow Stroma Cell Labeling

Although in our preliminary studies, we have used DAPI for cell labeling, retroviral vectors as the tool for



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cell labeling may also be employed. Likewise, other known techniques for cell labeling may be employed in accordance with the present invention. Retroviral vectors permit insertion of foreign synthetic genetic information in target cells. Reporter genes such as  $\beta$ -galactosidase, can therefore be stably integrated in chromosomal DNA. Expression of these transgenes permits ambiguous identification, by classic histochemical or fluorescence microscopy, of gene modified cells. As an example, retroviral labeling of hematopoietic stem cells is routinely carried out in the study of bone marrow transplantation in rodents. Undifferentiated (gene labeled) progeny cells of a widely different phenotype (lymphocytes, granulocytes, platelets, red blood cells) are readily detectable up to one year after bone marrow transplantation. We propose that retroviral labeled stroma cells that survive, proliferate or differentiate following cardiac implantation, will preserve and express the transgene as is classically observed in animals transplanted with retrovirus labeled hematopoietic stem cells. With this technique, important questions regarding bio-distribution of transplanted stroma cells can be addressed. Unambiguous identification of "labeled stroma cells", as well as their differentiated progeny, local and distant to the site of implantation in the injured heart, will become possible. Identification of other "unexpected" progeny cells, such as endothelial cells, interstitial cells, possibly others, will also be feasible. Furthermore, in accordance with an embodiment of the present invention sensitive PCR-based techniques were employed to detect as little as 1 to 100,000 transgene positive cells from tissue DNA extracts.



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According to an embodiment of the present invention cultured primary marrow stroma cells were readily transduced with synthetic retroviral vectors. In addition, high efficiency gene transfer in cultured stroma cells may 5 also be employed and genetically labeled cells can be expanded *in vitro* for up to 3 to 4 weeks without loss of reporter gene expression (in this case the green fluorescent protein). We have on hand a high titer VSV-G pseudotyped  $\beta$ -gal retroviral producer. We propose that 10 transplanted stroma cells genetically labeled with a  $\beta$ -galactosidase retrovector will be readily identified by classic X-Gal staining of cardiac tissue sections.

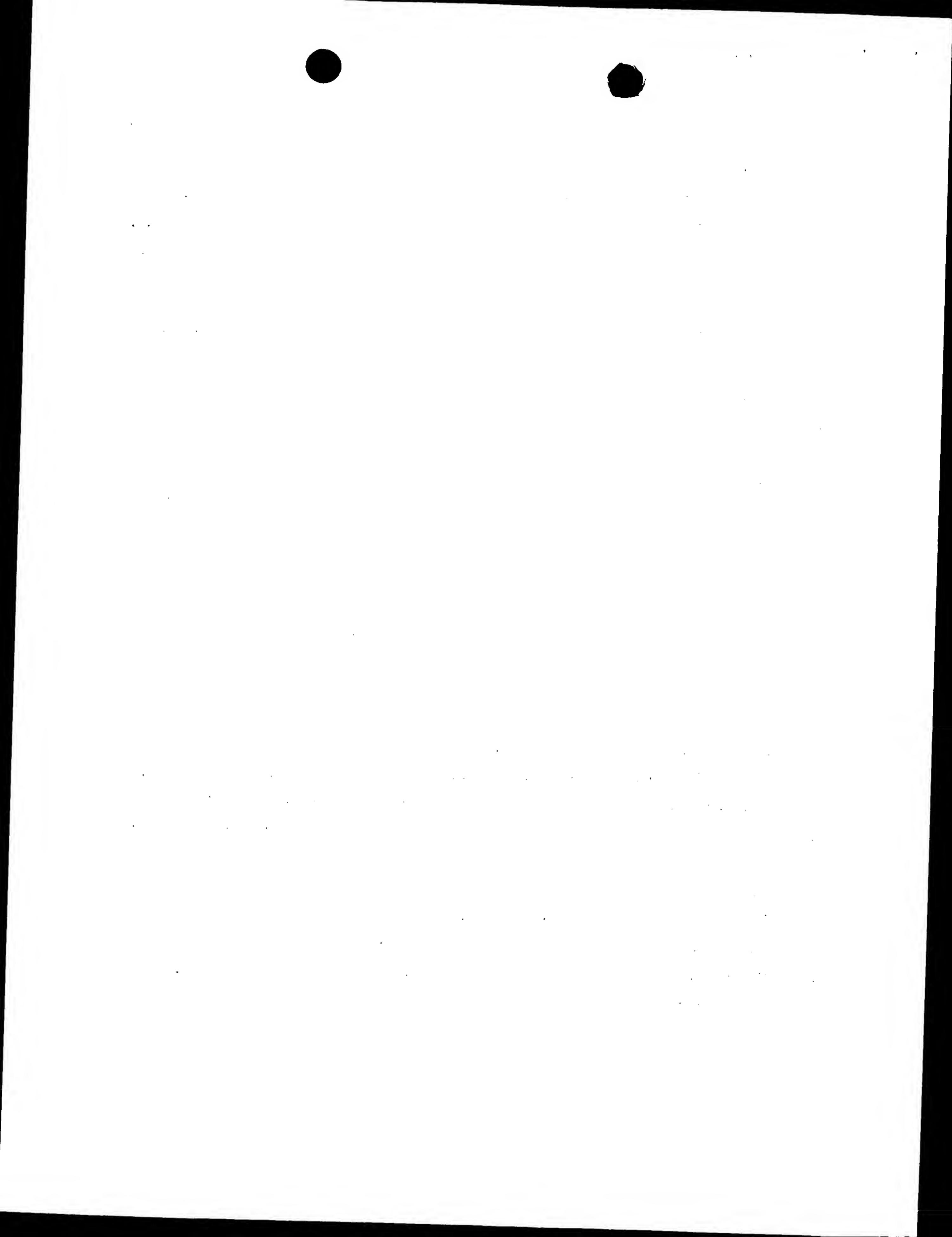
#### Coronary Artery Ligation Model in Rats

15 The recipient rats, both experimental and control, are isogenic Lewis rats weighing 175 to 200 grams. Anesthesia is induced and maintained with isoflurane (MTC Pharmaceuticals). The animals are intubated with an 18-gauge intravenous catheter and connected to a Harvard 20 rodent ventilator (Harvard Apparatus Co., Inc., South Natick, Mass.) at 85 breaths per minute. The heart is exposed via a 1.5 cm left thoracotomy incision. Under direct vision, using a 5-0 prolene suture, the anterior descending coronary artery which is visible in the 25 epicardium is ligated proximally. The thoracotomy is closed with 4-0 monofilament sutures. The muscle and skin layers are closed with 4-0 absorbable sutures and the animals are returned to their cages with filter tops. After the learning curve, the operative mortality is virtually nil.

30

#### Cell Transplantation into the Rat Heart

The recipient rats which underwent coronary artery ligation 2 weeks previously will undergo a second



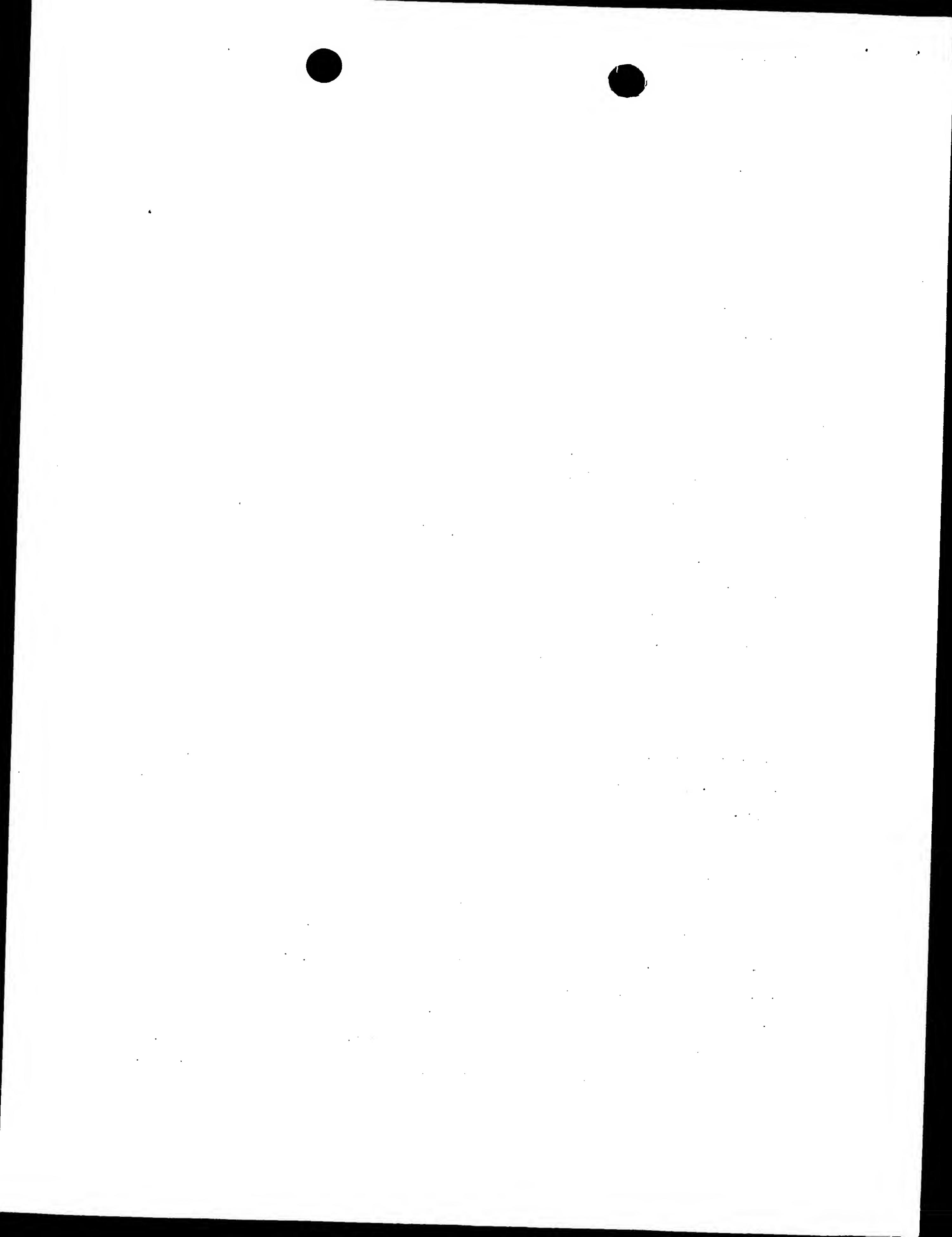
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operation. Anesthesia and thoracotomy will be performed in the manner described above. Under direct vision, the MSC suspension is injected into the lateral wall of the left ventricle with a 20-gauge needle, both at the center of the 5 ischemic segment of the myocardium, and at the border zone at the junction between the infarcted and normal myocardium. The thoracotomy closure and post-operative care are similar to that described above, and the animals sacrificed at intervals after this procedure, as stated 10 earlier.

The sham operated control rats will undergo an identical procedure as described for cell implantation in the experimental animals. The only difference is that instead of injecting cultured MSCs, an identical volume of 15 culture media (component described above) will be injected.

#### Echocardiographic Studies on Wall Motion

Transthoracic Doppler echocardiographic studies will be performed in the rats every week following implantation. 20 The rats are anesthetized as described above, the chest wall shaved, and echocardiography performed using our echo system equipped with a 7.5 MHZ transducer (Hewlett Packard Sonos 2500). A 2-dimensional short axis view of the left ventricle is obtained at the level of the papillary muscle 25 to record M-mode tracing. Anterior and posterior end-diastolic and end-systolic wall thickness and LV diameters are measured using the American Society of Echocardiography Lineage Method, from at least three consecutive cardiac cycles. The changes in wall thickness and ventricular 30 segmental wall motion and diameter will be recorded on videotape, and assessed by blinded independent echocardiographers.



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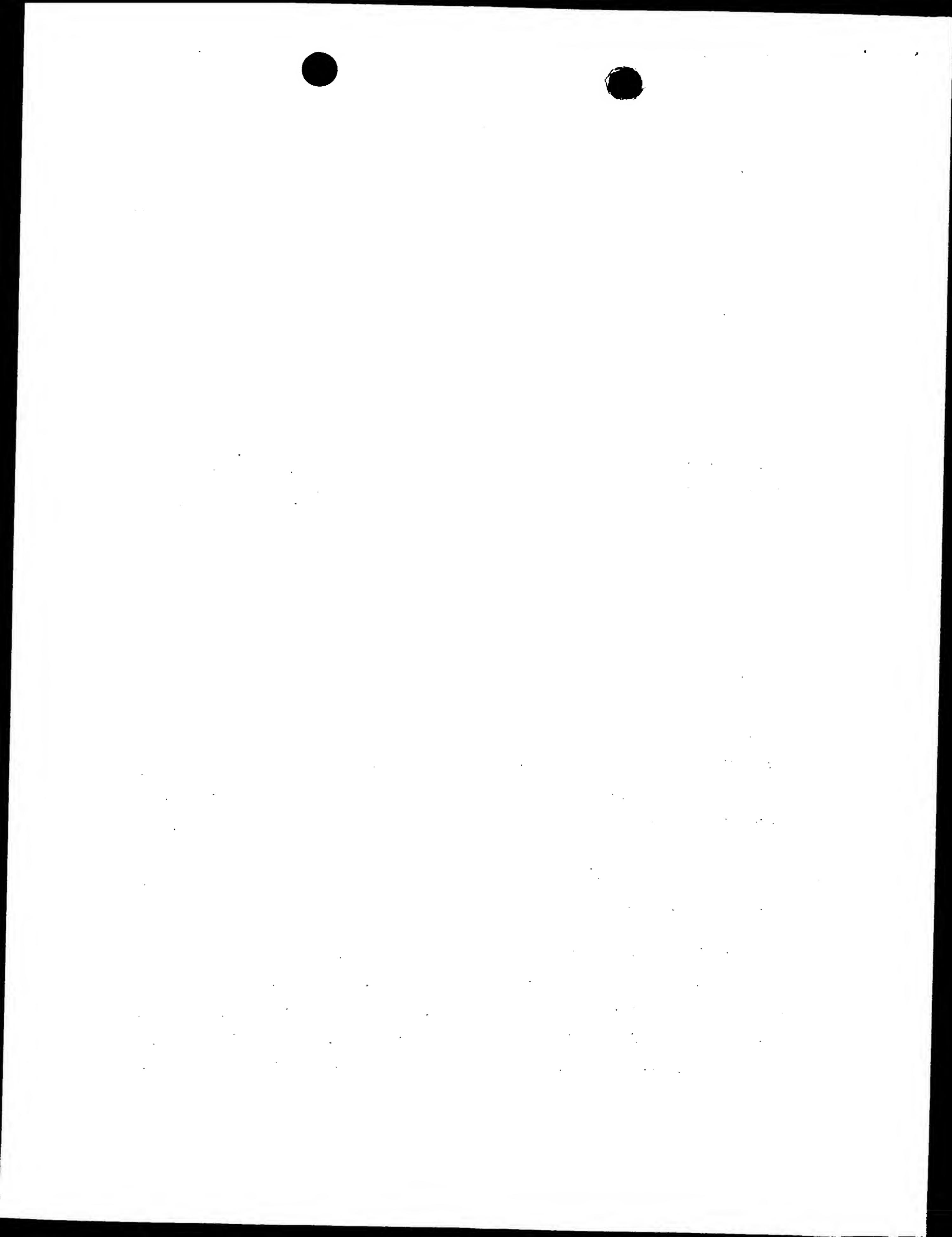
#### Morphological and Immunohistochemical Studies

The heart specimens obtained from the recipient rats at various intervals will be perfused with 100 ml of saline through the posterior wall of the left ventricle, avoiding 5 the transplant area, then processed for frozen sections. The lateral wall of the left ventricle is isolated from the remainder of the heart. Sections 6  $\mu\text{m}$  thick are cut from the hearts and successive sections collected by gelatine coated glass slides. This ensures that different stains could be 10 applied on successive sections of the tissue cut through the transplanted area (Figs. 1 and 3). One of the sections is mounted and stained with X-Gal, to identify and view the  $\beta$ -gal labeled donor cells. An adjacent section is stained with hematoxylin and eosin as described in the 15 manufacturer's specification (Sigma Diagnostics) to depict nuclei, cytoplasm and connective tissue. Other adjacent sections will be immunolabeled using various antibodies for immunohistochemical evaluation in order to identify phenotypic expression at the molecular level. These 20 antibodies include those against myosin-slow molecules, cardiac gap junctional protein Connexin 43, desmin, and sarcomeric myosin (MF 20). Finally, specimens will also be processed and sent for ultrastructural examination in our future studies.

25

#### Example II

Bone marrow stromal cells (MSCs) were infused into ascending aorta of isogenic recipient rats after coronary artery ligation. MSCs were found to traffic through the 30 coronary circulation to the injured heart, and form cardiomyocytes, fibroblasts and endothelial cells etc., depending on the specific microenvironment. Thus, autologous MSCs appear to participate in the post-infarct



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repair and remodeling process, and provide a therapeutically utility in treating heart failure and in improving cardiac function.

## 5 Methods

### Animals

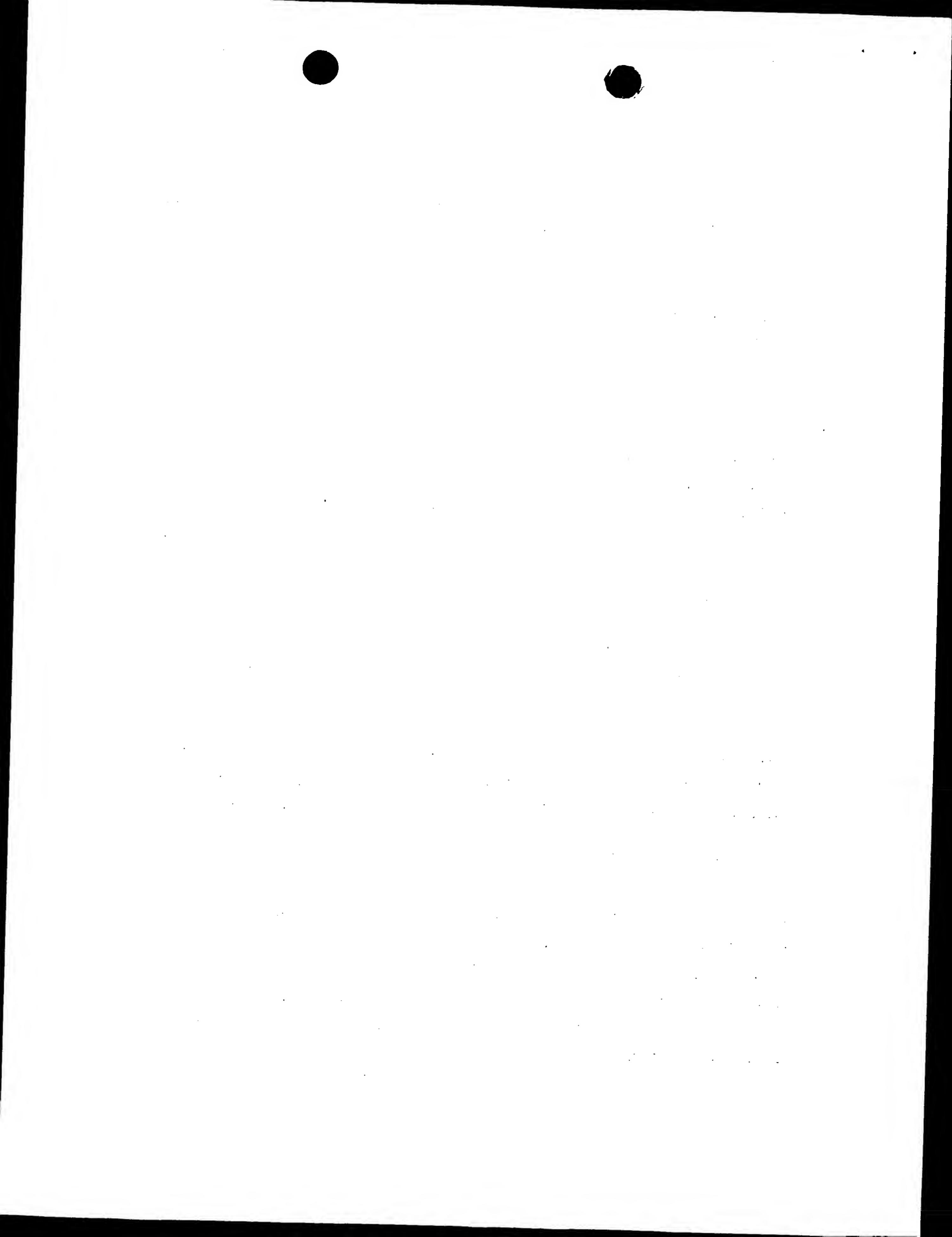
Male inbred Lewis rats 200 to 250 gm were obtained from Charles River Laboratories. These isogenic rats were used as donors and recipients to simulate the autologous infusion of MSCs in the future clinical application. All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996 and the "Guide to the Care and Use of Experimental Animals" of the Canadian Council on Animal Care.

### Myocardial Infarction Model

12 recipient rats were anesthetized with isoflurane (MTC Pharmaceuticals). Rats were intubated and ventilated at 85 breaths/min. The heart was exposed via left thoracotomy incision. The left coronary artery was identified and ligated proximally using a 7-0 polypropylene suture. Regional myocardial ischemia was confirmed by the rapid occurrence of akinesia in the area at risk. The wound was then closed.

### Isolation and Culture of Marrow-derived Stromal Cells

Isolation and primary culture of MSCs from the femoral and tibial bones of donor rats were performed according to Caplan's method (Wakitani S, Saito T, Caplan A.I. Myogenic cells derived from rat bone marrow



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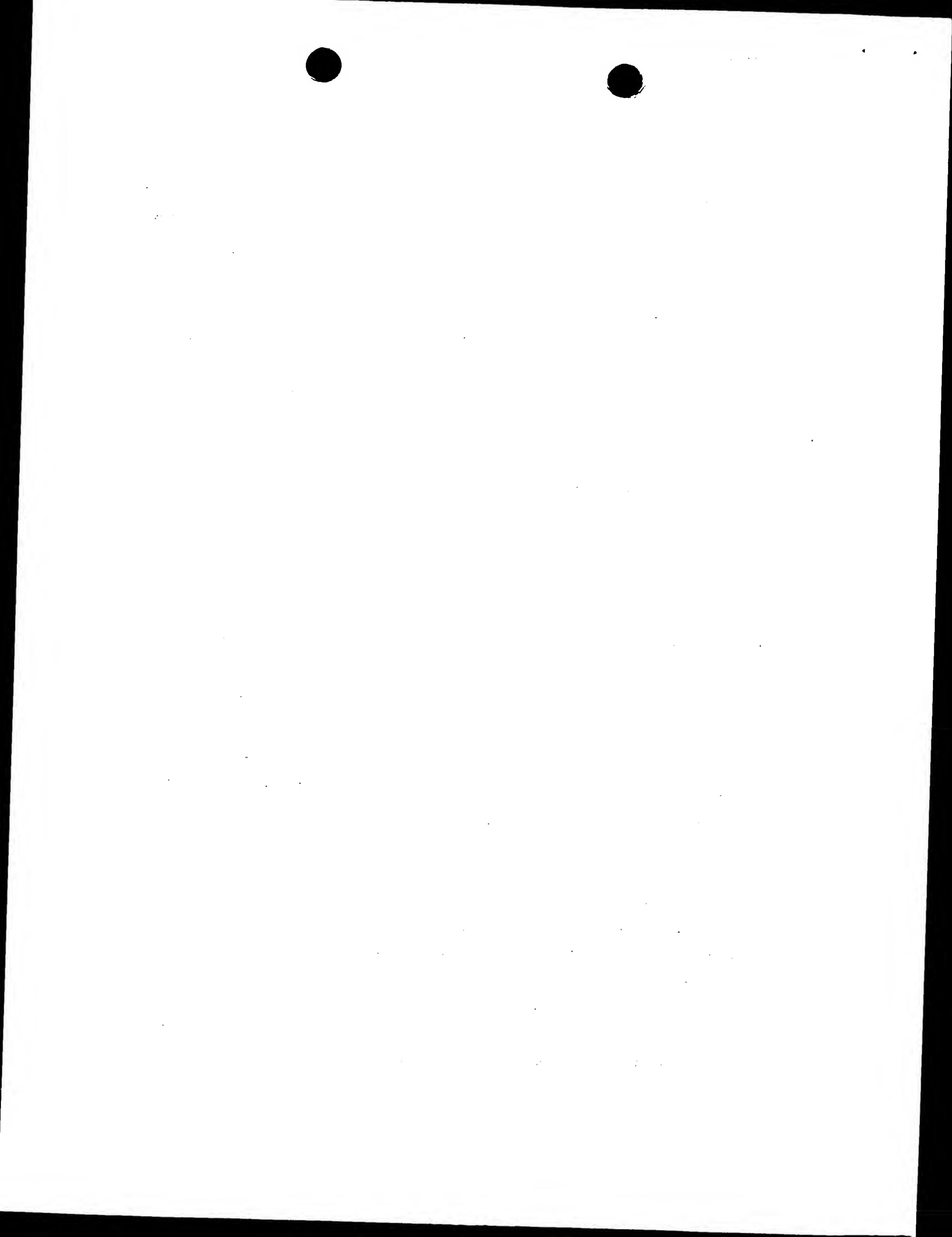
mesenchymal stem cells exposed to 5-azacytidine. Muscle & Nerve 1995;18:1417-1426). After overdose with pentobarbital (100 mg/Kg given intraperitoneally), the femoral and tibial bones were collected. Both ends of the bones were cut away  
5 from the diaphyses. The bone marrow plugs were hydrostatically expelled from the bones with complete medium. The marrow plugs were disaggregated and the dispersed cells were centrifuged and resuspended twice in complete medium. These cells in 10 mL of complete medium  
10 were then introduced into tissue culture dishes. Medium was completely replaced every 3 days and the non-adherent cells discarded. Each primary culture was replated twice (first and second passages) to three new plates when the cell density within colonies became 80% to 90% confluent. After  
15 the twice-passaged cells became nearly confluent, they were harvested and used for the coronary infusion experiments.

#### Medium

The cells were routinely cultured in complete medium consisting of Dulbecco's modified Eagle's medium (DMEM)  
20 containing selected lots of 10% fetal calf serum and antibiotics (100 U/mL penicillin G, 100 $\mu$ g/mL streptomycin and 0.25 $\mu$ g/mL amphotericin B; all obtained from Gibco laboratories) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

#### Marrow-derived Stromal Cell Labeling

25 P+E86 murine ectropic retrovirus-packaging cells, which are derived from NIH 3T3 mouse fibroblasts, were obtained from Dr. Denis Cournoyer (McGill University, Montreal, QC, Canada) (Momparler RL, Laliberte J, Eliopoulos N, Beausejour C, Cournoyer D. Transfection of murine fibroblast cells with human cytidine deaminase cDNA confers resistance to cytosine arabinoside. Anti-Cancer Drugs 1996;7:266-274). The GP+E86 cells were transfected



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with the purified plasmid DNA pMFG-LacZ in a 10:1 molar ratio using the standard calcium phosphate transfection kit (Pharmacia, Baie d'Urfe, Quebec, Canada). The LacZ gene encodes for the production of bacterial  $\beta$ -galactosidase.

5 These cells were plated at 25% confluence for 48 hours. The second passaged MSCs growth medium was replaced with the supernatant from the GP+E86 cells (containing the replication-defective retrovirus carrying the  $\beta$ -gal reporter gene) to transfect the MSCs overnight and then replaced

10 with normal complete medium for the following day. After three times of transfection, MSCs were then collected (approximately  $2 \times 10^6$  cells for one infusion) and resuspended in 50 $\mu$ L of serum-free DMEM and stored on ice until infusion into the ascending aorta. Some culture

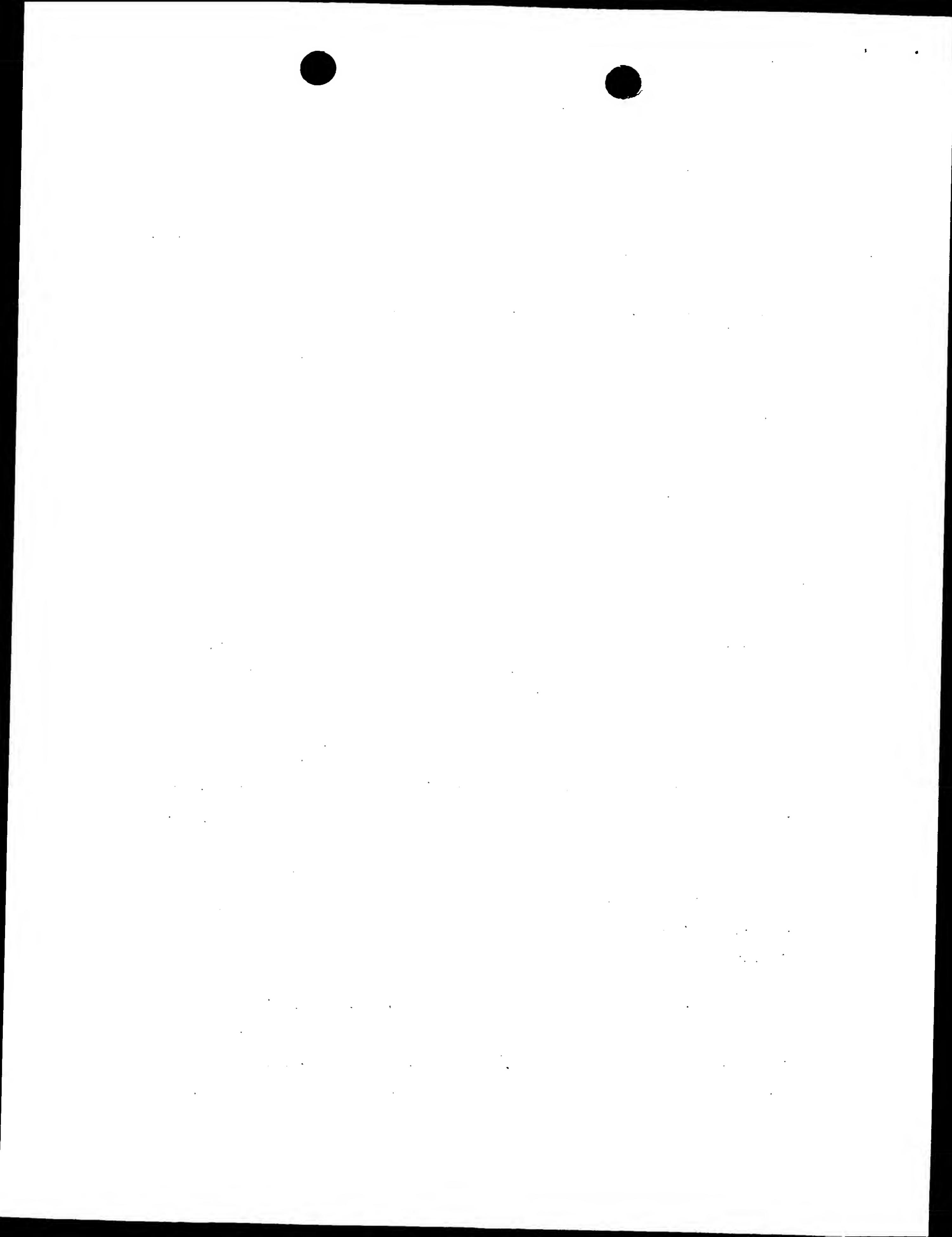
15 plates were selected for histochemical staining *in vitro* for  $\beta$ -galactosidase activity. The cells were fixed in 2% formaldehyde and 0.2% glutaraldehyde in phosphate buffered saline (PBS) in 4°C for 5 minutes. Staining for  $\beta$ -gal was accomplished at 37°C for 16 hr in a solution containing 1

20 mg/ml 5-bromo-4-chloro-3-indoyl- $\beta$ -D-galactoside (X-gal), 2% dimethylsulfoxide, 10 mM potassium ferricyanide, 10 mM potassium ferrocyanide, 1 mM magnesium chloride, and 0.02% Nonidet P-40 in PBS, pH7.3.

#### Infusion of Marrow-derived Stromal Cells

25 Two weeks after the coronary ligation, the 12 recipient rats were prepared for infusion of MSCs. Anesthesia was induced and maintained as above. The rats were intubated and connected to the ventilator. The ascending aorta was exposed through upper median sternotomy

30 and looped after dissection. Under direct vision, transfected MSCs suspension was then infused into the



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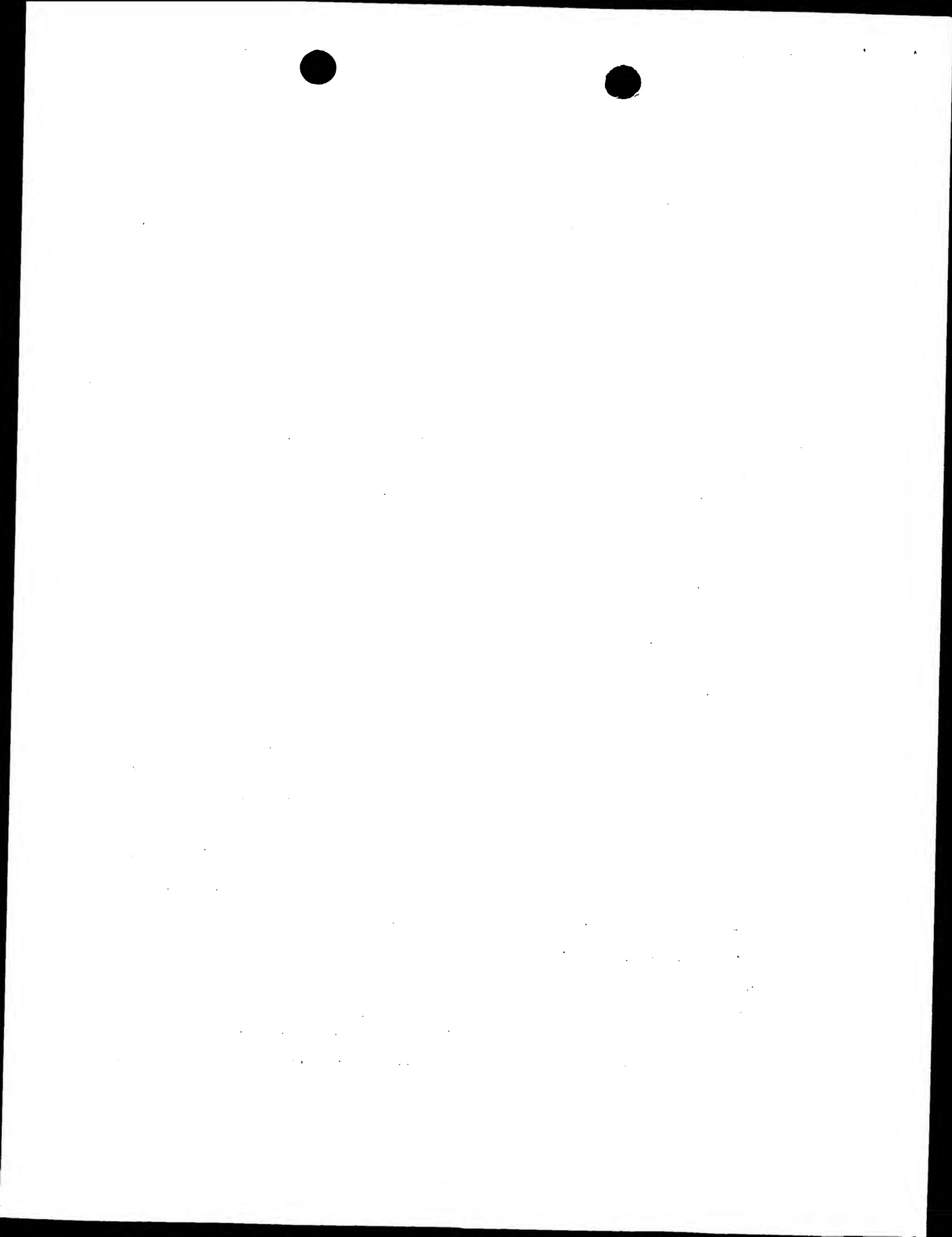
briefly distally clamped ascending aorta (about 20 seconds). After the infusion, the puncture bleeding site over ascending aorta was controlled by compression with gauze. The wound was then closed in layers.

5      Histology and Histochemical Staining for  $\beta$ -galactosidase Activity

Two rats were sacrificed immediately after the infusion. The hearts were excised and sliced along short axis of left ventricle to 3 mm thick sections in series, 10 and fixed in 2% paraformaldehyde in PBS for 2 hours. The sections were then cryoembedded after protection with 20% sucrose in PBS overnight. The other 10 rats were taken for their final experiments 4 weeks after the MSCs infusion. After overdose with pentobarbital, the hearts were exposed 15 and injected with 100 ml saline (0.9%) through the apex of the left ventricle, then perfusion-fixed with 2% paraformaldehyde in phosphate buffered saline (PBS). The hearts were excised, sliced and prepared as mentioned above. Cryosections 6 $\mu$ m in thickness were collected in each 20 3 mm section sample across a set of gelatin-coated glass slides. One of every 10 cryosections was collected for histochemical staining for  $\beta$ -galactosidase activity as mentioned above. The sections were then counter-stained 25 with hematoxylin and eosin. Tissue sections were examined with an Olympus microscope (BX-FLA, Olympus). Digital images, transferred to a computer equipped with Image Pro software (Media Cybernetics, MA), were subsequently printed.

Histochemical Stain for Gap-junction Protein, Connexin 43

30      The gap junctions which constitute cardiac muscle specific intercalated discs were demonstrated by histochemical stain for connexin 43, using rabbit anti-



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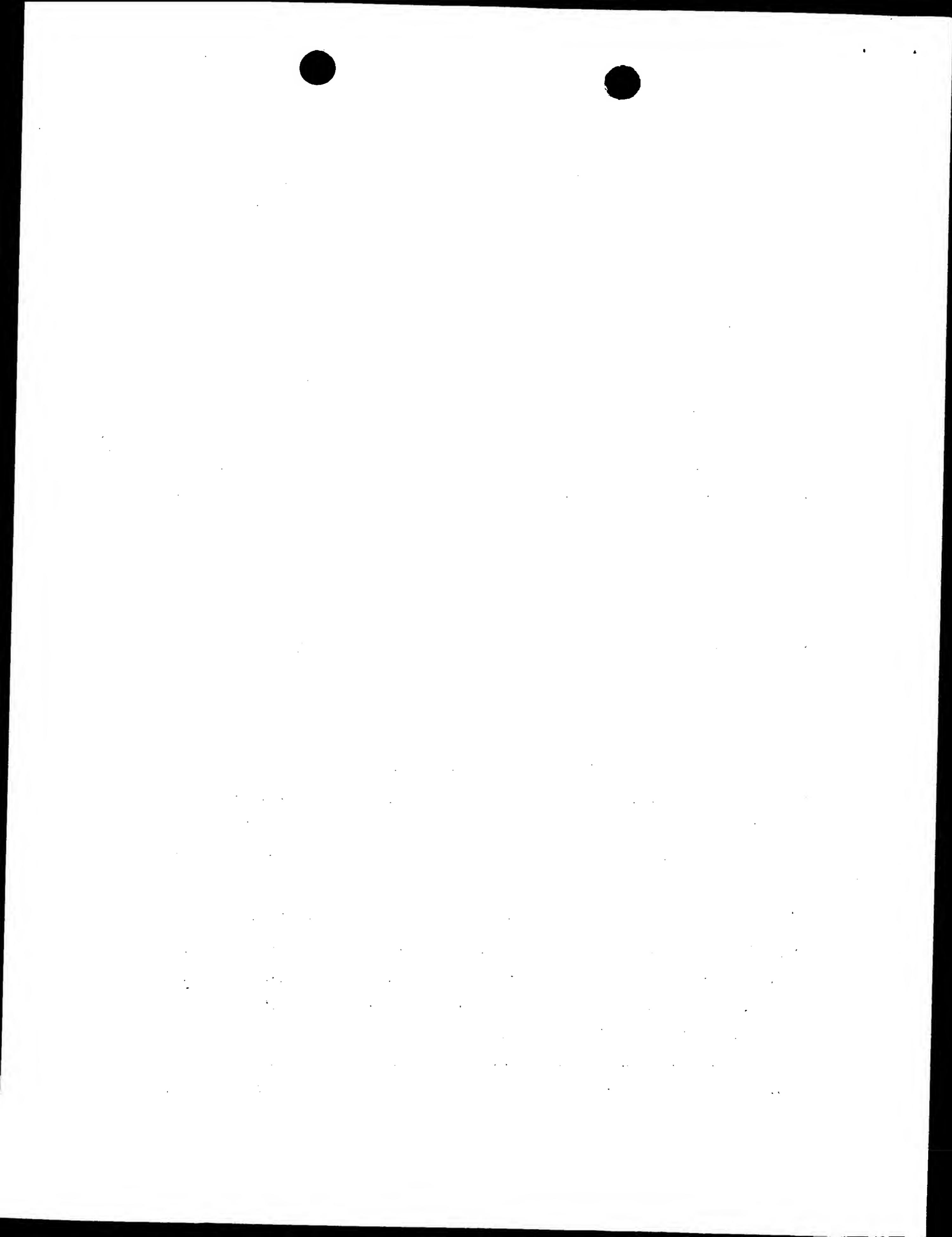
connexin antibodies (Zymed Laboratories Inc., San Francisco). Diaminobenzidine was used as a chromogen to produce the brown colour which represent gap junctions linking cardiomyocytes together (Nagy JI, Li WE, Roy C,  
5 Doble BW, Gilchrist JS, Kardami E, Hertzberg EL. Selective monoclonal antibody recognition and cellular localization of an unphosphorylated form of connexin 43. *Exp. Cell Res.* 1997; 236:127 - 136).

### Results

10 Cultured MSCs were observed by phase microscope to assess the level of expansion and to verify the morphology at each culture medium change. Most of the hematopoietic stem cells were not adherent to the culture plate and removed with changes in medium. The adherent cells were  
15 seen as individual cells or colonies of only a few cells on day 6; however, they replicate rapidly and form colonies of up to 100 cells after the first week of culture. By the end of second week, the colonies of adherent cells have expanded in size, with each colony containing several  
20 hundred to several thousand cells. Adherent MSCs from rat legs have similar morphology, most being fibroblastic in appearance, with a few adipocytic, polygonal cells (Fig. 4). This phenotype was retained throughout repeated passages under nonstimulating condition.

25 We transfected twice-passaged MSCs with replication-defective retrovirus carrying the  $\beta$ -gal reporter gene as cell labeling before their implantation. The transfected MSCs showed clear histochemical staining for  $\beta$ -galactosidase activity (Fig. 5). Transfection efficiency of the MSCs  
30 culture was almost 100%.

The rats were sacrificed at the following intervals after the MSCs infusion: immediately for 2 rats; and 4

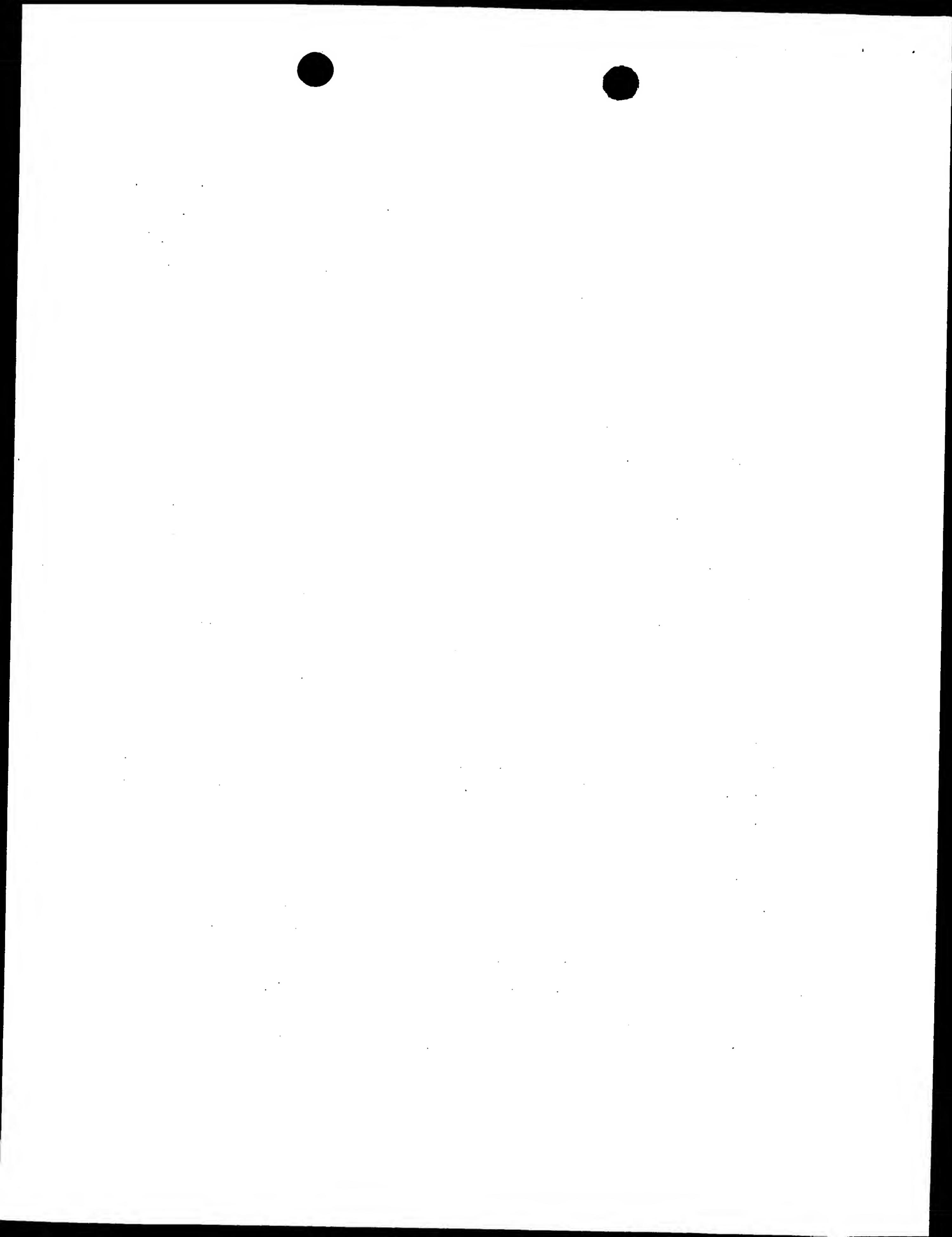


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weeks for 10 rats. Gross examination of the excised hearts shows clear myocardial scar formation (about 40 % of LV free wall) on all 12 recipient rats. Cryosections of the specimens were selected (as detailed in Methods) for  
5 histochemical staining of  $\beta$ -galactosidase activity to trace and evaluate the morphology and phenotype changes of infused MSCs. Labeled cells could be identified in both rats sacrificed immediately after MSCs infusion, and in 6 out of 10 rats sacrificed 4 weeks later.

10 Immediately after MSCs infusion,  $\beta$ -gal positive cells were consistently found in all selected sections to be trapped within the coronary capillaries surrounded by endothelial cells all over the non-infarct area (Fig. 6). We failed to identify any  $\beta$ -gal positive cells in the  
15 infarction zone at this time. Four weeks after MSCs infusion, some  $\beta$ -gal positive cells could be found within the normal myocardial area outside the infarction scar (Fig. 7). They have centrally located nuclei and are connected among themselves and with surrounding host  
20 cardiomyocytes ( $\beta$ -gal negative cells) by intercalated disk-like structure, which are characteristics of normal cardiomyocytes. However, the  $\beta$ -gal positive cells also could be detected individually or in clusters within the myocardial scar (Fig. 8). They appear unorganized and  
25 scattered in the infarction scar with fibroblast-like morphology, similar to that of the surrounding  $\beta$ -gal negative (host) fibroblast cells. Some  $\beta$ -gal positive cells were found incorporated into endocardium (Fig. 9) and coronary capillary endothelium within or outside the  
30 infarct scar area (Figs. 10A & 10B).

Tissue sections showing labeled cells with histological features of cardiomyocytes (as in Fig. 7) were



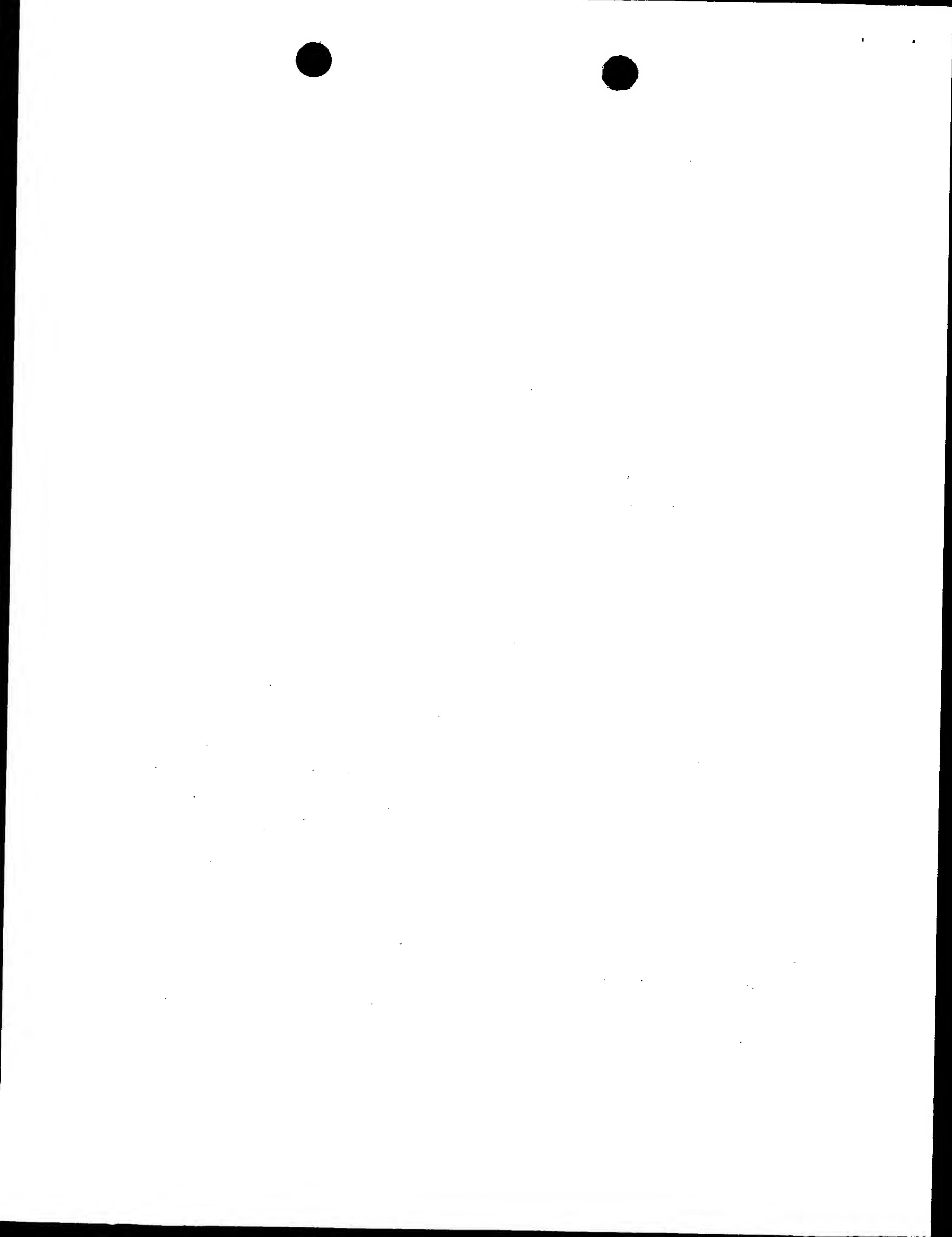
further studied immunohistochemically using antibodies against connexin 43, a major constituent protein of gap junctions in the intercalated discs of cardiac myofibers. The demonstration of such junctional structure (Fig. 11) 5 further confirms the phenotype of the differentiated labeled cells, and their integration into the native cardiac myofibers.

#### INDUSTRIAL APPLICABILITY

According to the present invention, MSCs are 10 determined to traffic through the circulatory system to the injured heart, and are capable of forming cardiomyocytes and other types of cells, depending on the specific microenvironment. In addition, endothelial progenitor cells in the MSCs population may be involved in the post- 15 infarction neovascularization process. As a result, MSCs display myocardial cell differentiation properties *in vivo* and provide a promising therapeutic use in improving myocardial healing following infarction. Labeled cardiac myocytes and fibers were present in the implant site, which 20 exhibited positive immunohistochemical stains in normal or immature cardiac myocytes. These findings indicate that the use of autologous marrow stroma cells (MSCs) in transplantation for myocardial regeneration in animals and humans.

25 In addition, the present invention has application in the area of experimental research, where by MSCs may be employed in a variety of *in vivo* animal models to further study the influence of the micro environments on stem cell differentiation, and on cellular signaling mechanisms.

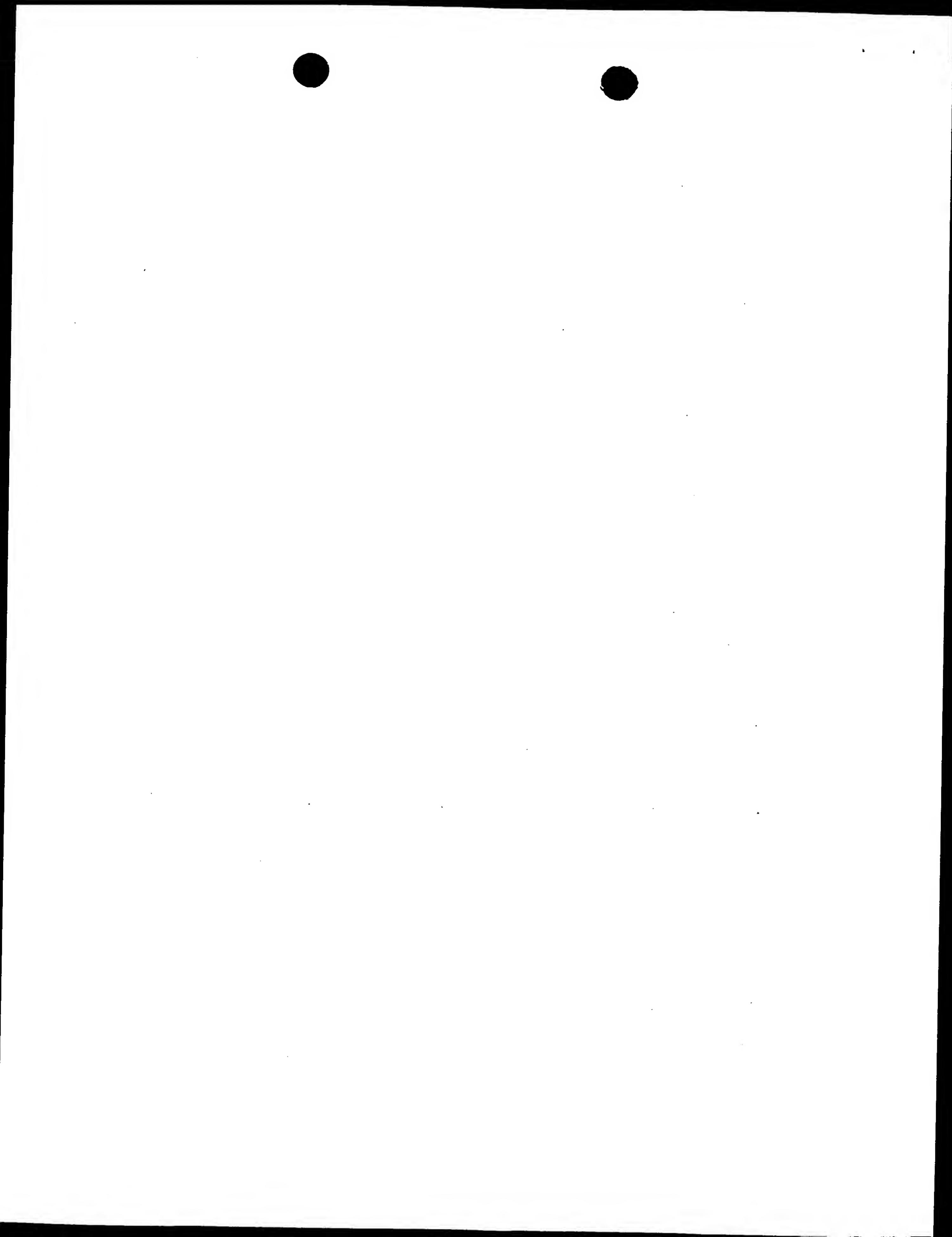
30 Further echocardiographic studies are expected to demonstrate improved systolic thickening of the ischemic ventricular wall segment, and reduced ventricular size and



remodeling, as had been reported following the implantation of other donor cells.

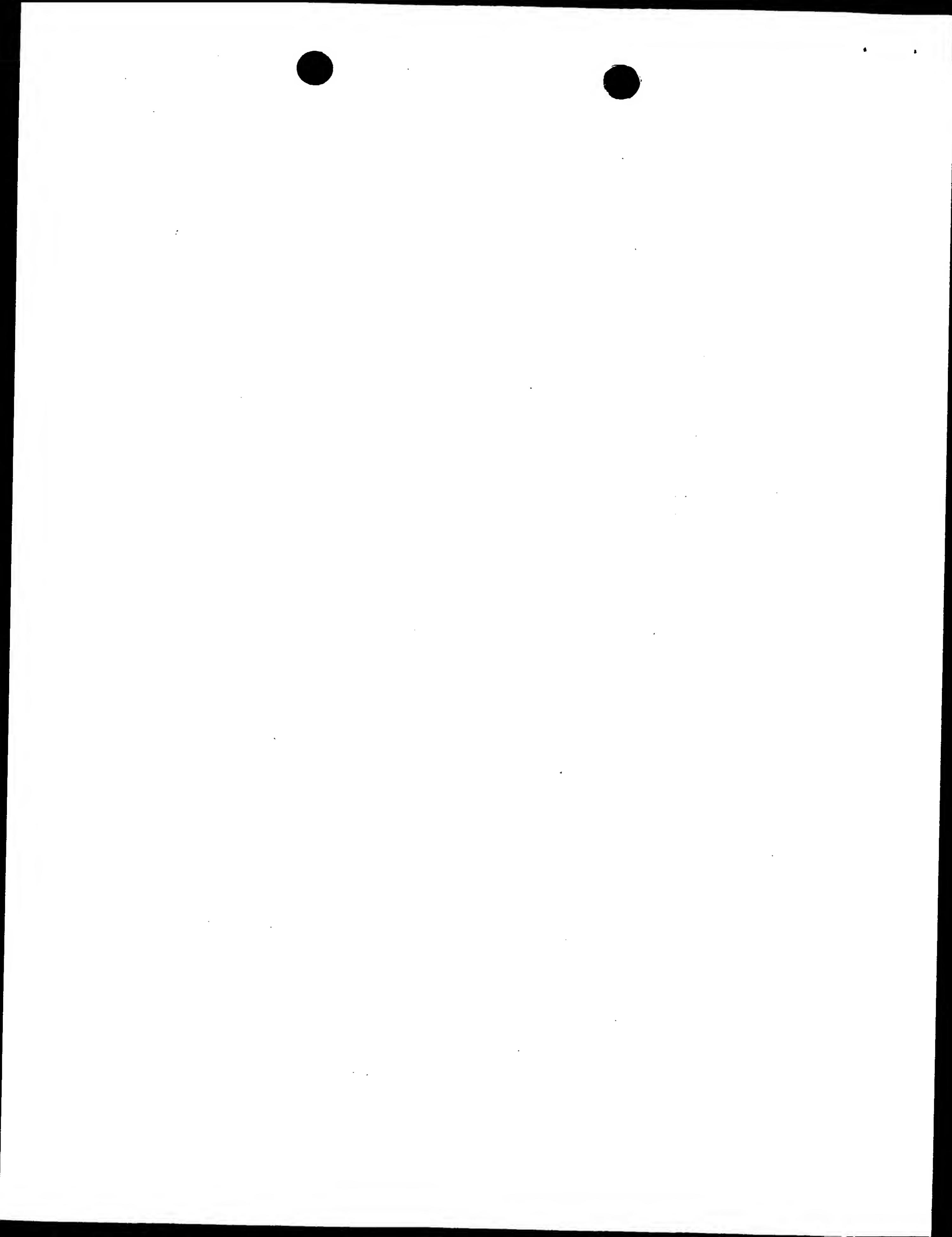
Future studies include clinical trials and mechanistic investigations employing the teachings of the present invention. Minimal medical and ethical difficulties are expected for clinical trials encompassing the present invention for the numerous reasons herein described. It is fully contemplated that the findings of the present invention will offer a valuable basis to pursue such scientific knowledge. For example, a comparision of the differentiation of labeled cells implanted at the center, and at the periphery of the infarcts will be made. In addition the micro-environments responsible for MSC differentiation in the myocardium will be further explored. Differentiation may be facilitated at the peripheral border zone, if direct cell-to-cell contact is an important signaling mechanism for such differentiation to take place. The role of cytokines and other growth factors will also be examined in the future. Further still, methodologies for transplanting autologous MSCs in patients to improve cardiac function will be optimized in future studies.

While the invention has been described in connection with specific embodiment thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses or adaptations of the invention following in general, the principles of the invention and including such departures from the present disclosure as come within the known customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.



## WHAT IS CLAIMED IS:

1. A method of improving cardiac function in a patient with heart failure without eliciting an immune response and without sacrificing the patient's skeletal muscle; which comprises the step of transplanting autologous bone marrow stroma cells (MSCs) into said patient's myocardium to grow new muscle fibers.
2. The method of claim 1, which further comprises the step of using cell labeling technique to confirm survival and differentiation of implanted MSCs, and to identify said MSCs phenotype by both morphology and molecular markers.
3. The method of claim 1, which further comprises examining the effects of the micro-environment of implanted MSCs on their differentiation and phenotype expression.
4. The method of claim 1, which further comprises examining functional contribution of MSCs implanted into an ischemic segment of the myocardium.
5. The method of claims 1 to 4, wherein said transplanting is effected in the myocardium *in situ*, in the myocardium artery or using a catheter from within the myocardium.
6. The method of claims 1 to 4, wherein said transplanting is effected in association with angiogenesis factors.
7. Use of autologous marrow stroma cells for improving cardiac function, wherein said autologous



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marrow stem cells are introduced *in situ* into a myocardium.

8. The use as claimed in claim 7 wherein said marrow stroma cells differentiate into myocardial specific cell types in microenvironments within the myocardium.

9. The use as claimed in claim 8 wherein said marrow stroma cells differentiate in the microenvironments of the myocardium into cardiomyocytes, fibroblasts or endothelial cells.

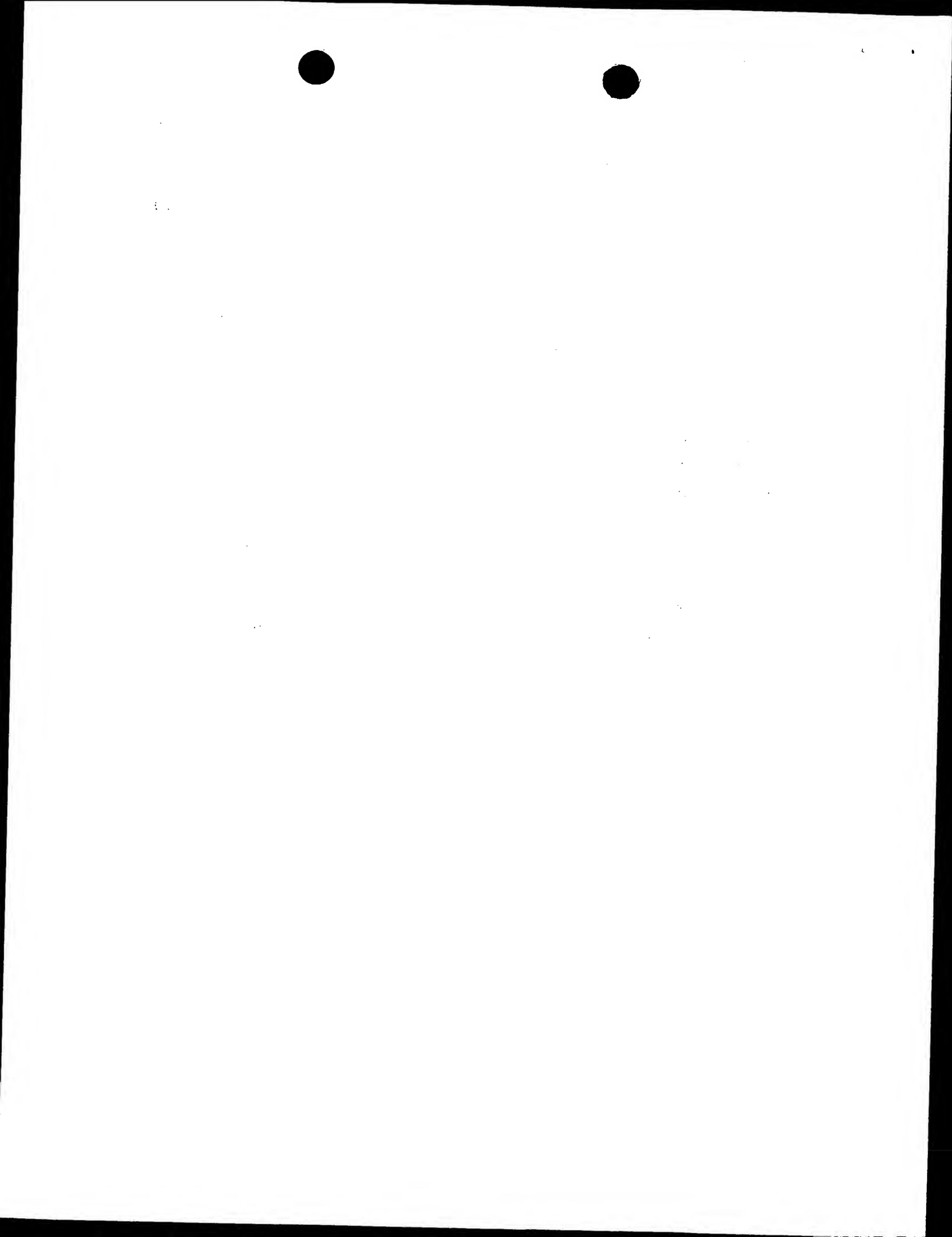
10. The use according to claim 7 wherein said marrow stroma cells are delivered by selective infusion into the myocardium.

11. The use according to claim 10 wherein said selective infusion is intra-coronary delivery of the marrow stroma cells.

12. The use according to claim 8 wherein said marrow stroma cells are delivered to said microenvironments by coronary circulation.

13. A method of treating cardiac failure, said method comprising:

- (a) retrieving bone marrow from a patient suffering from cardiac failure;
- (b) isolating marrow stroma cells from said bone marrow;
- (c) expanding said marrow stroma cells in culture; and
- (d) transplanting said marrow stroma cells into a myocardium of said patient.



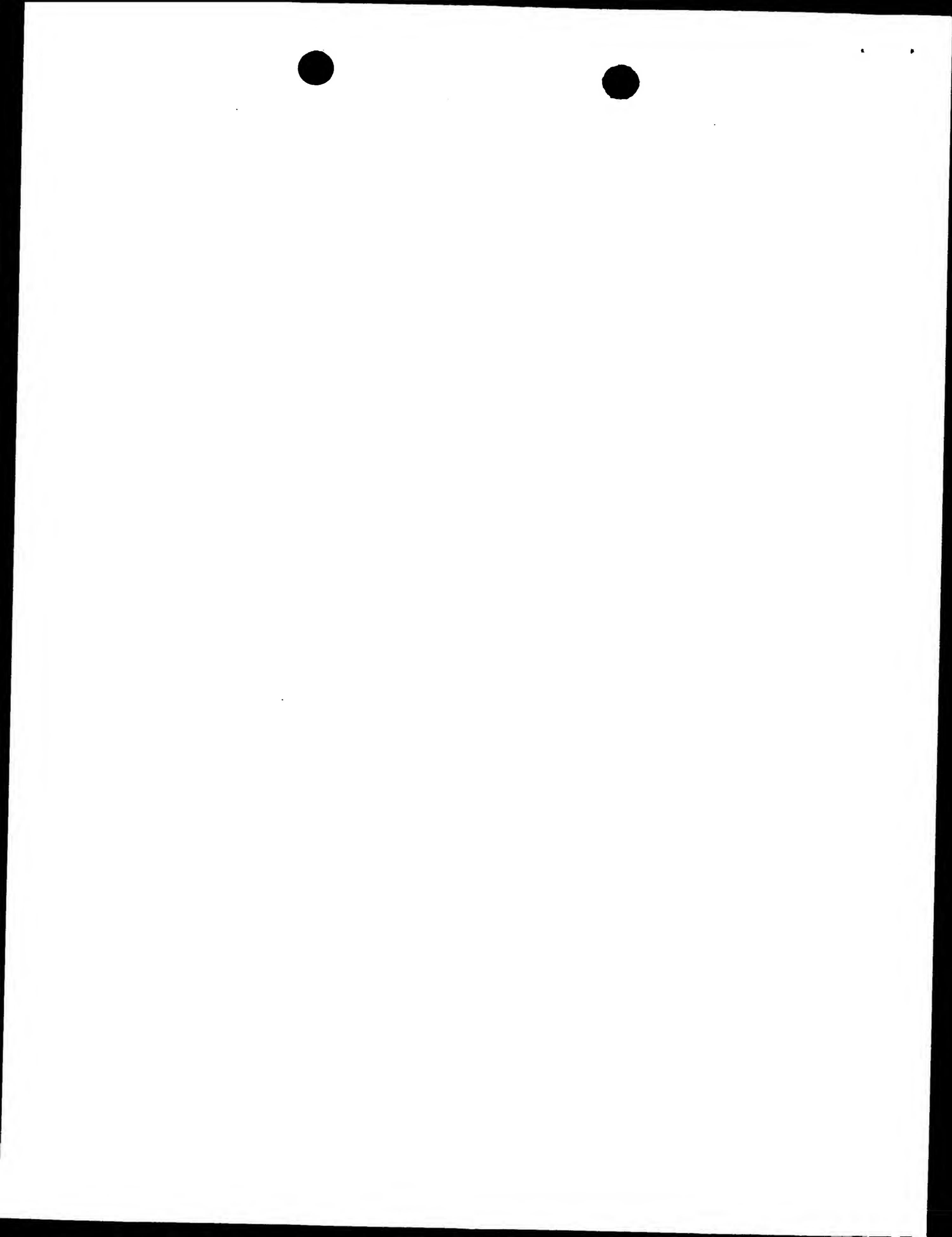
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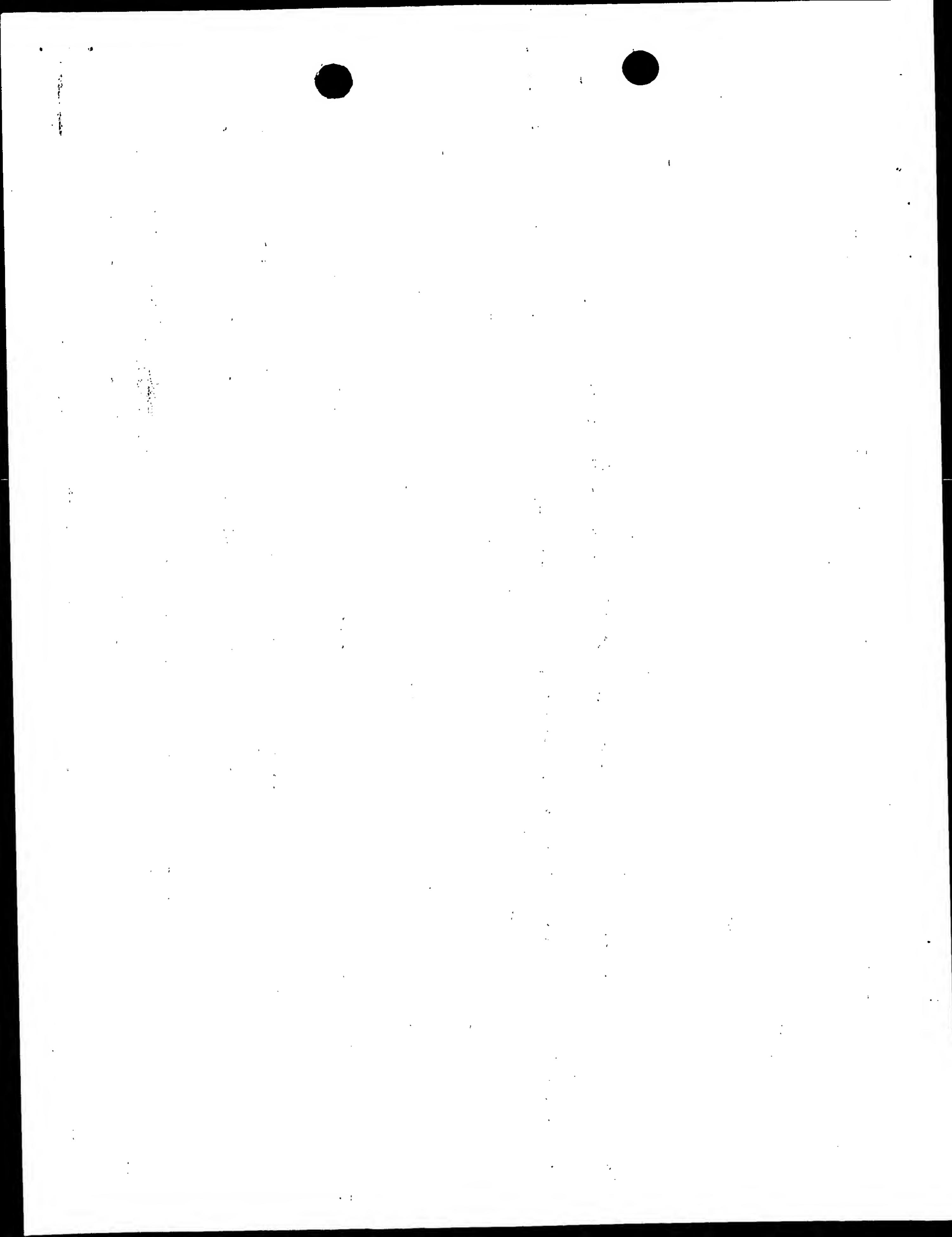
14. The method of claim 13 wherein said step of retrieving bone marrow includes performing a bone marrow puncture.

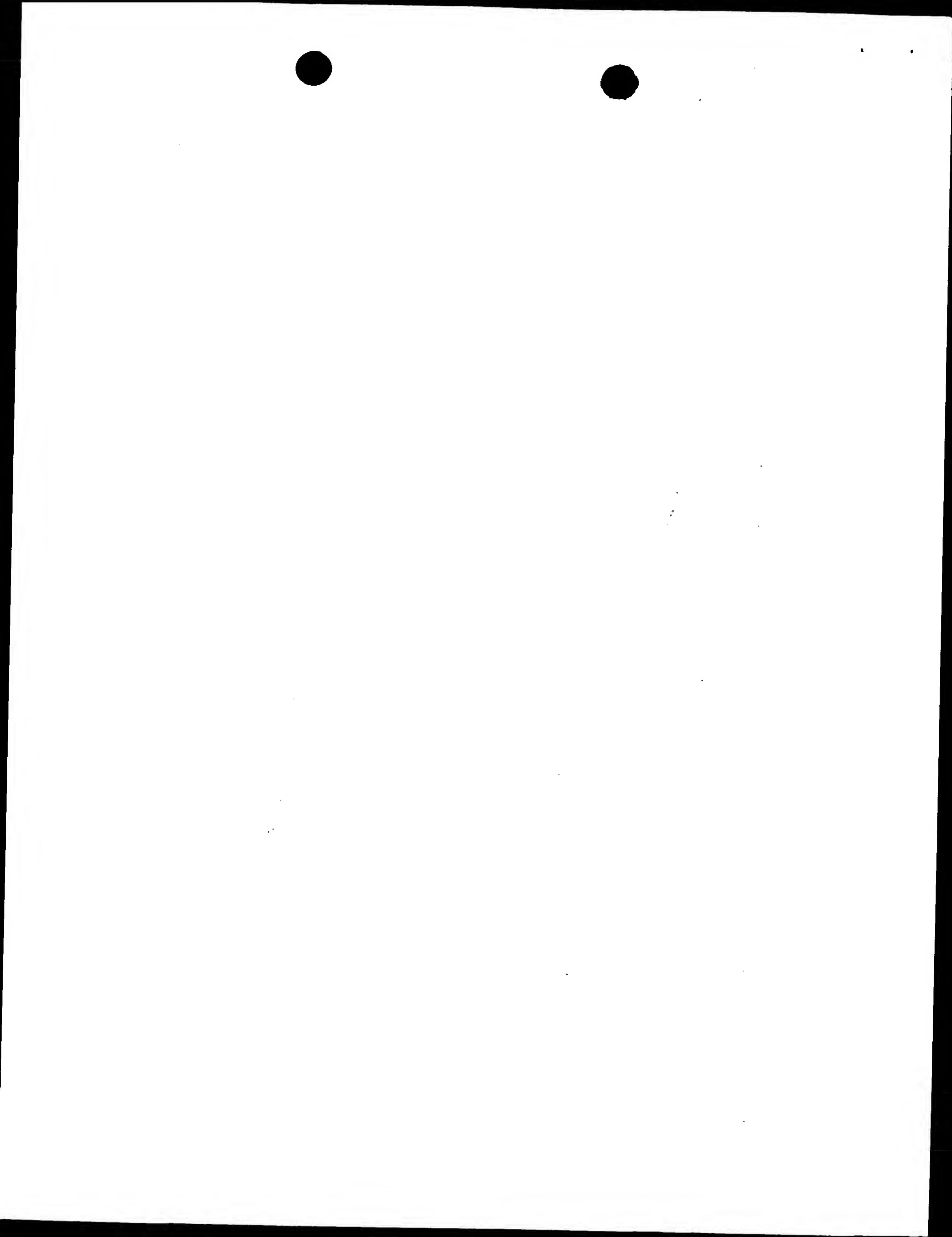
15. The method of claim 13 wherein said step of transplanting said marrow stroma cells into the myocardium includes selective infusion of said cells into coronary circulation.

16. The method of claim 13 wherein said step of transplanting said marrow stroma cells into the myocardium is achieved by transvenous catheter injection.

17. Use of autologous marrow stroma cells for examining the effects of a myocardial micro-environment on marrow stroma cell differentiation, wherein said autologous marrow stem cells are introduced *in situ* into an ischemic segment of a myocardium of an animal model.







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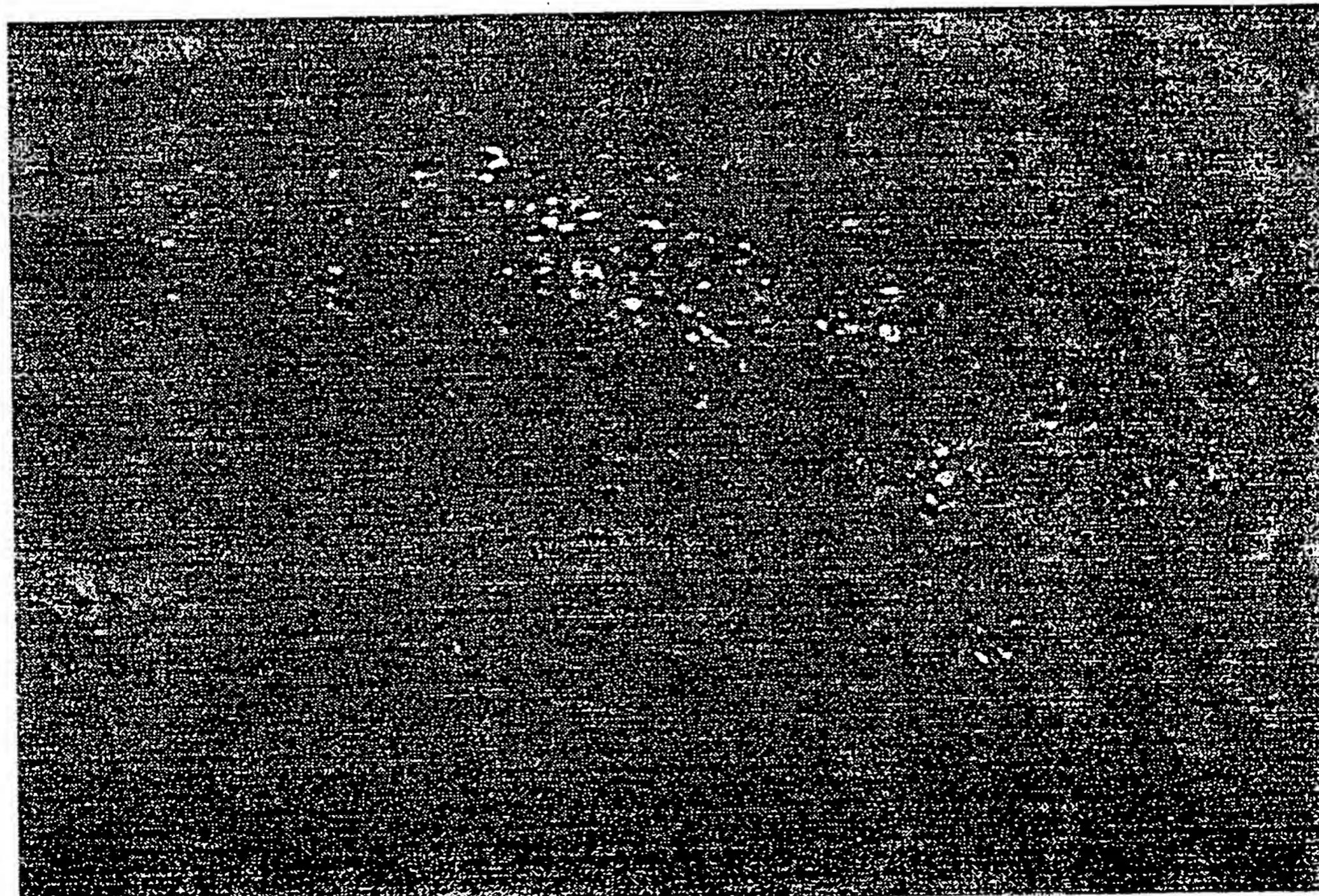
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**FIG. 1A**



**FIG. 1B**

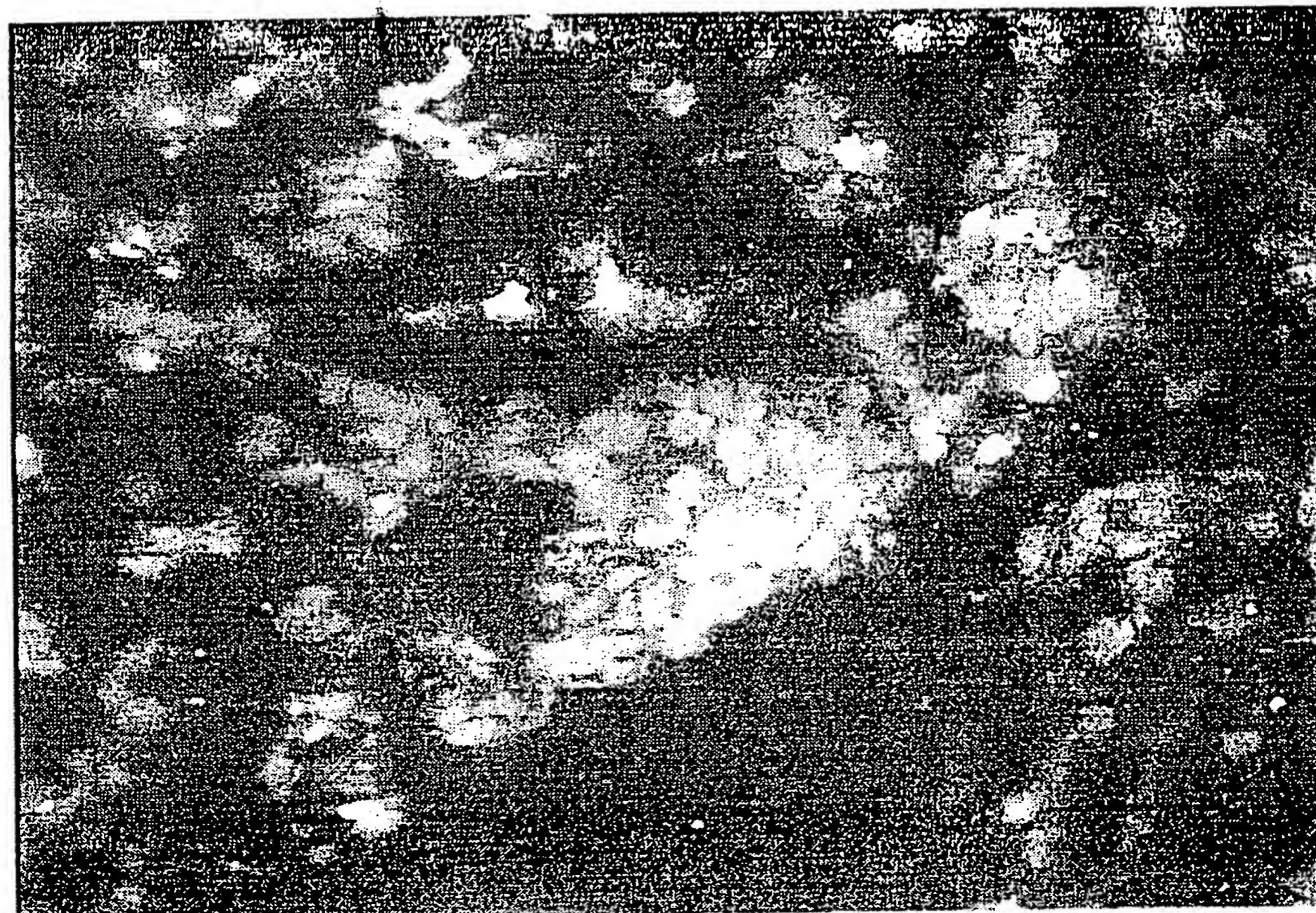
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**FIG. 2A**



**FIG. 2B**

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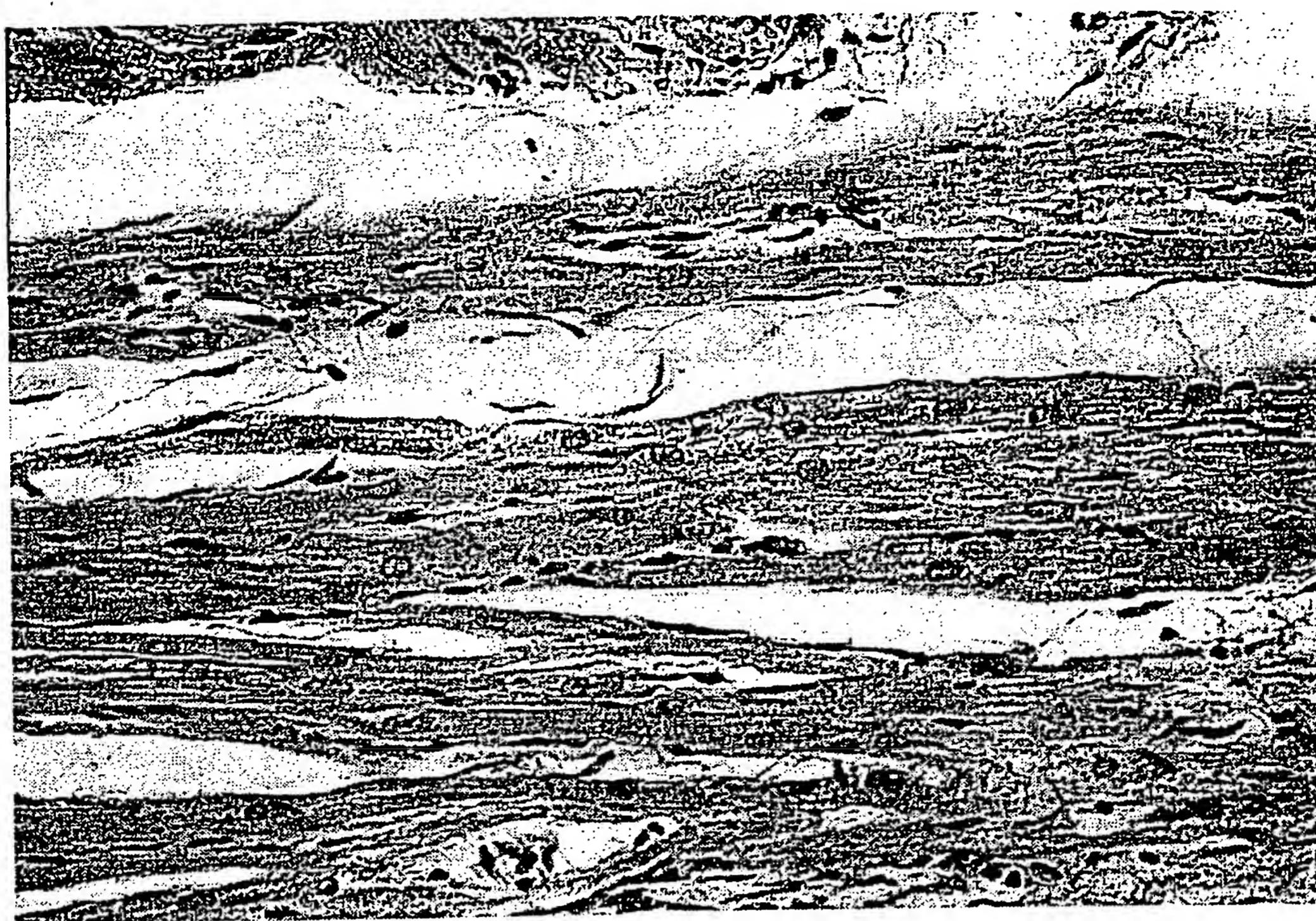
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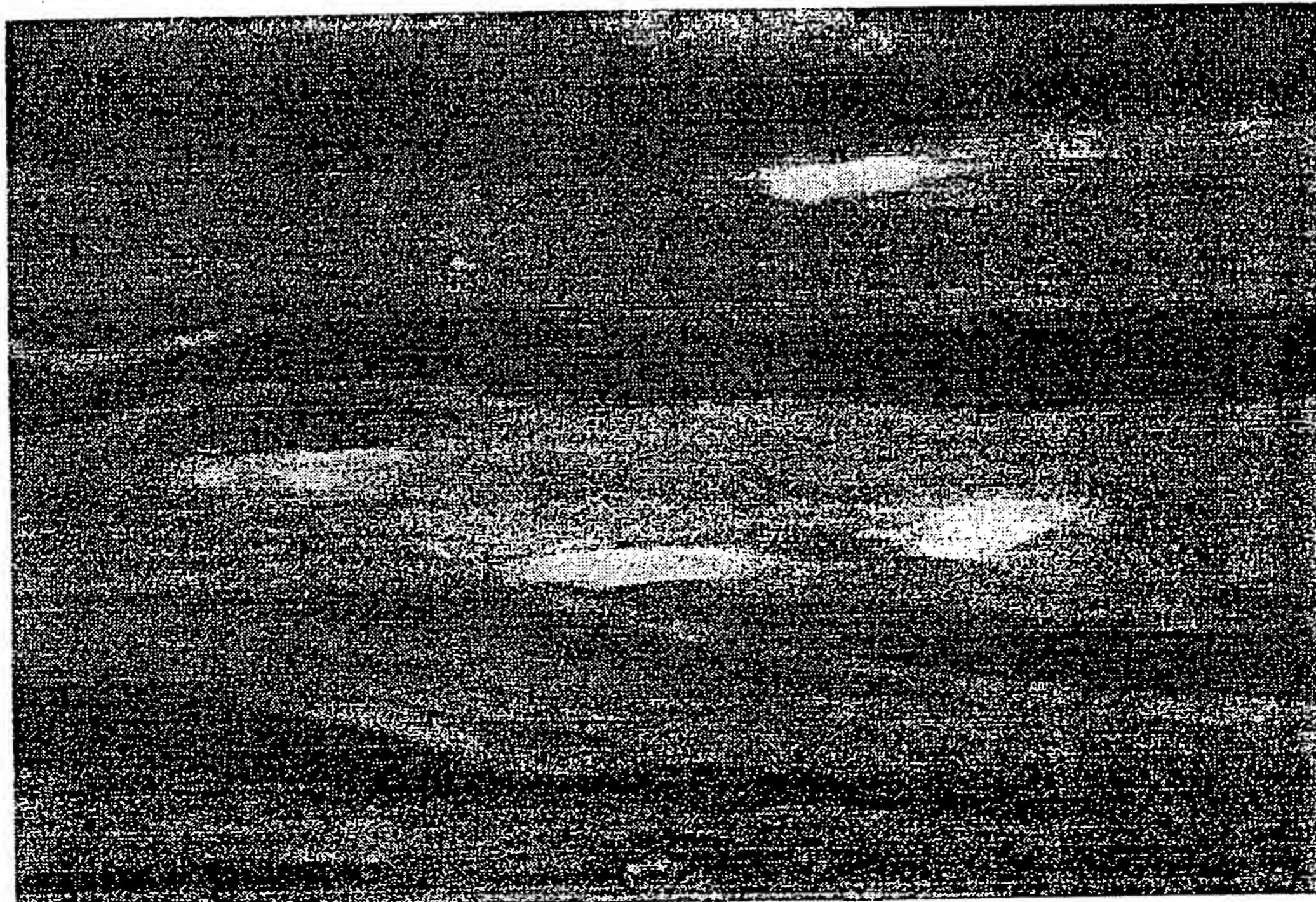
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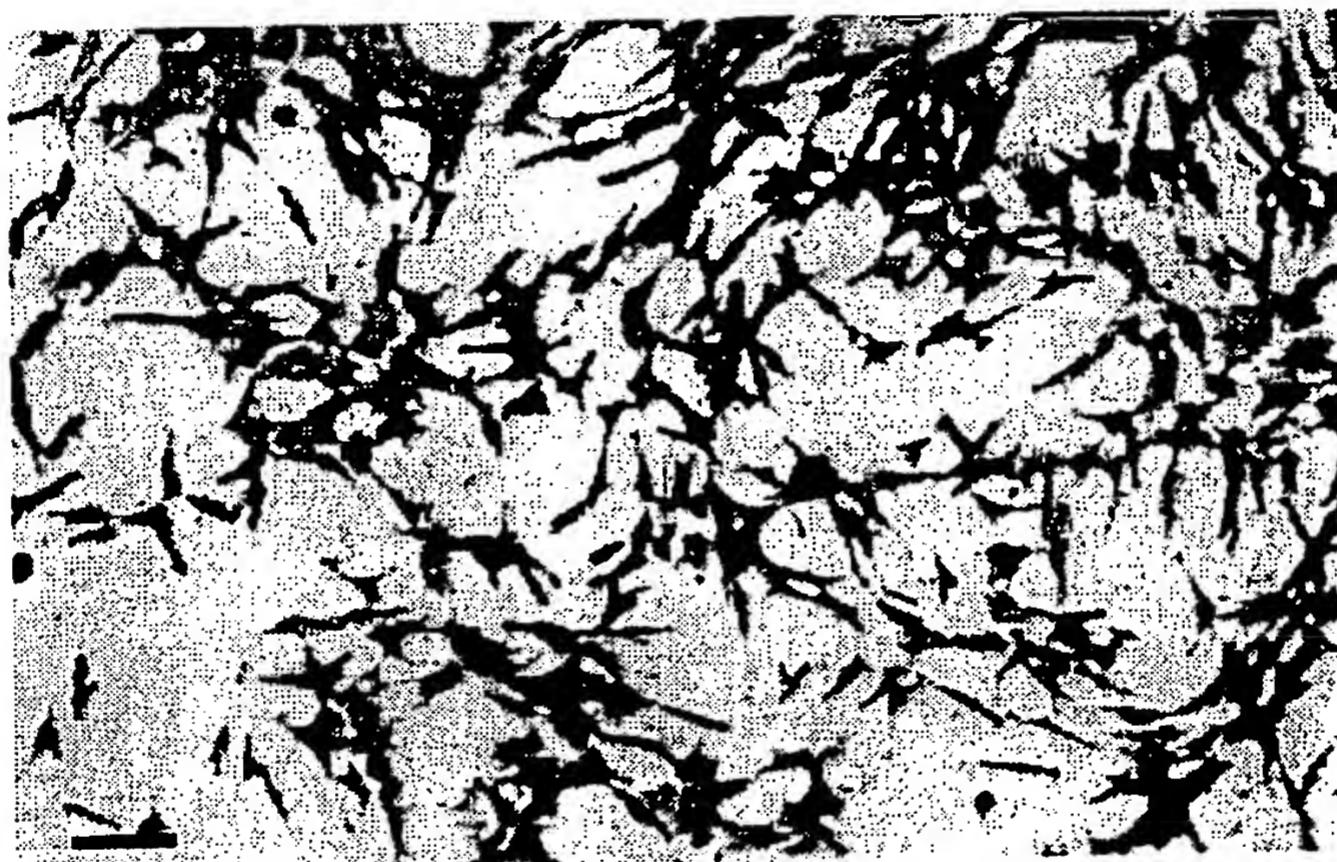
**FIG. 3A**



**FIG. 3B**  
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**FIG. 4****FIG. 5****FIG. 6**

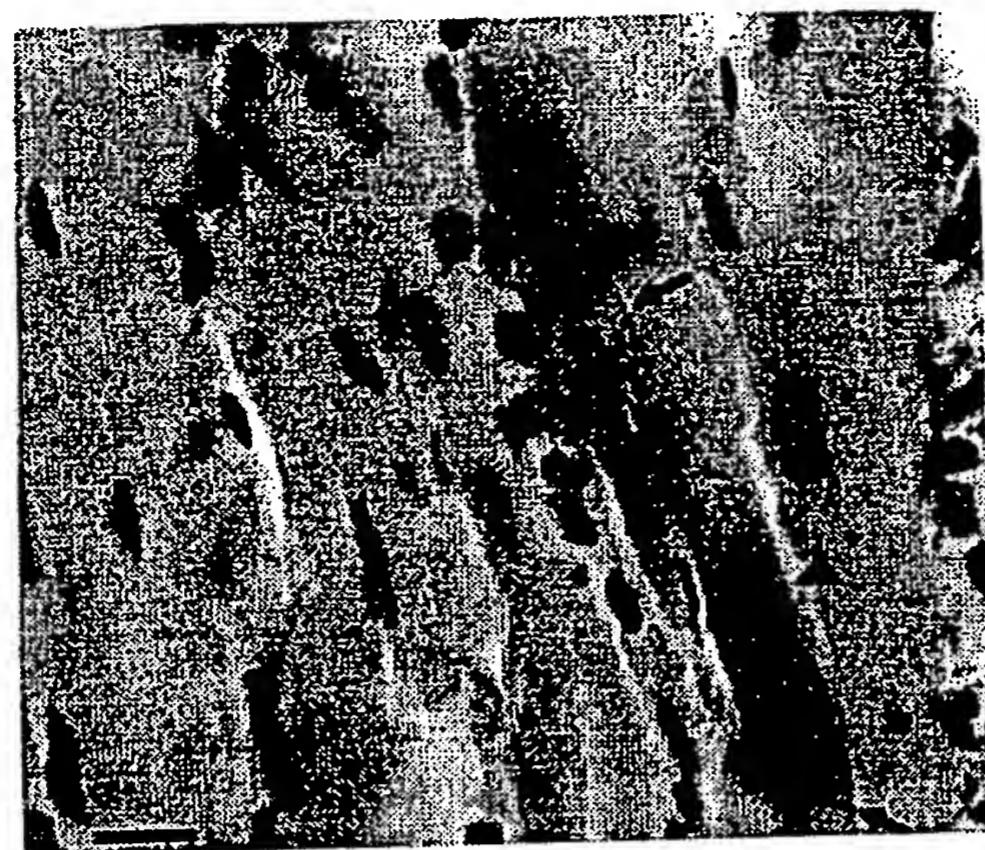
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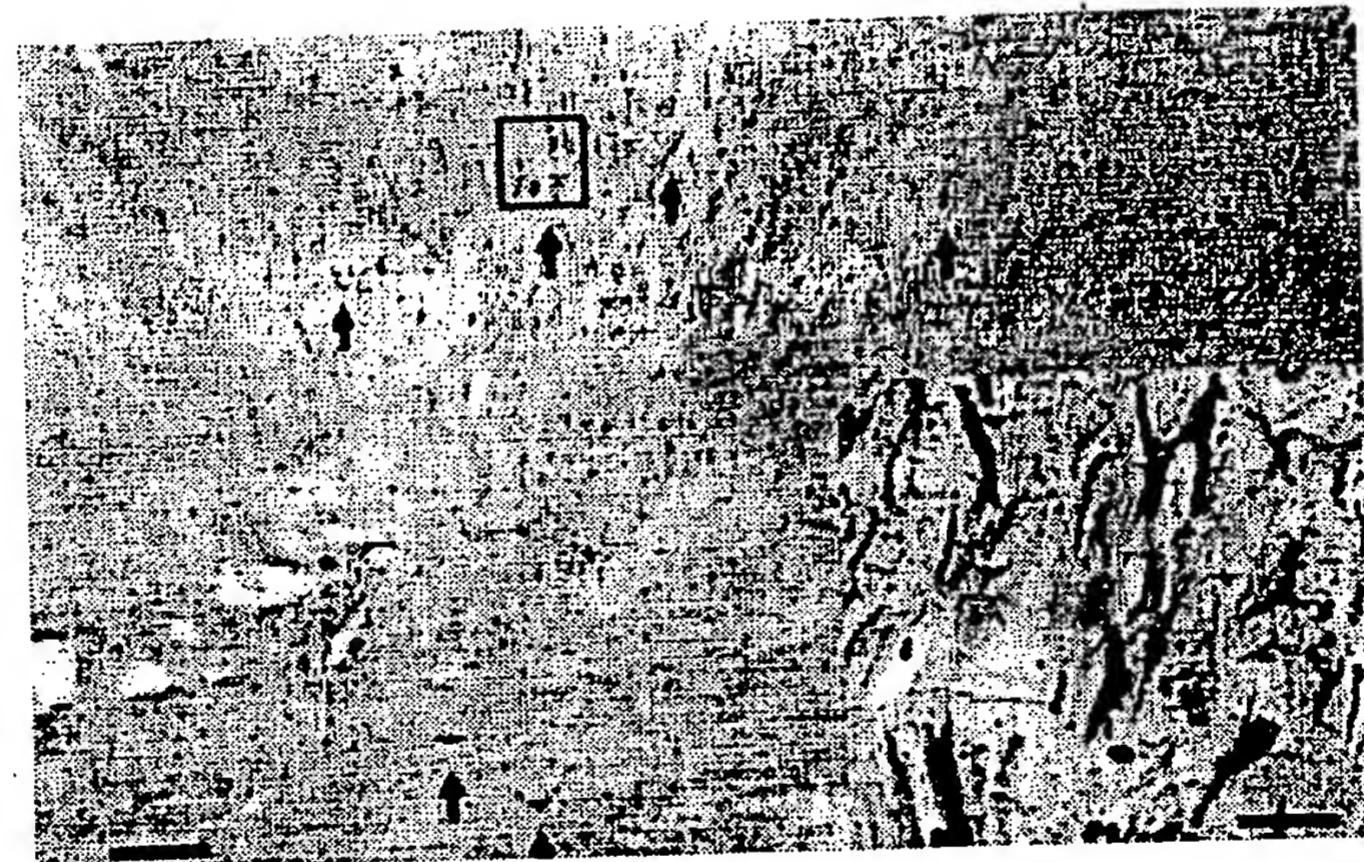
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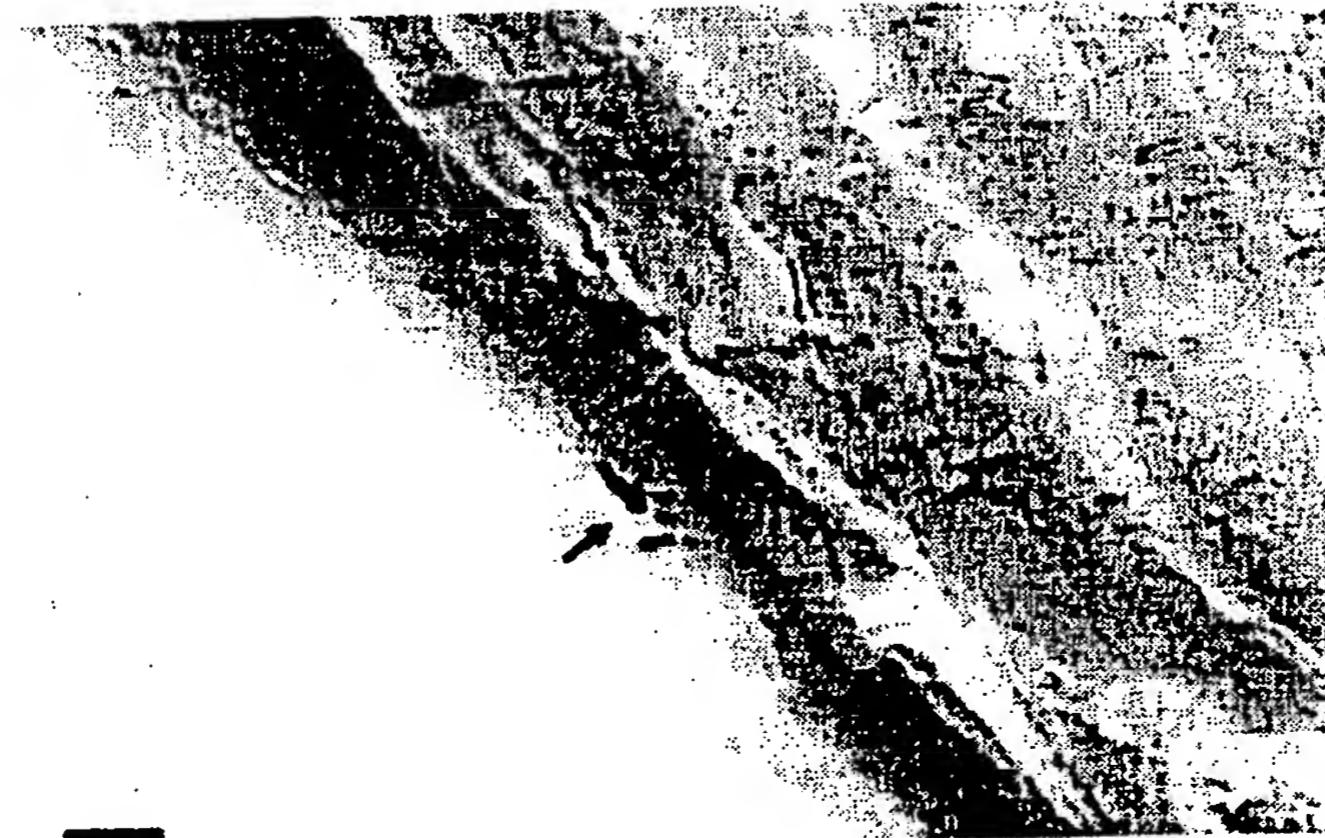
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**FIG. 7**



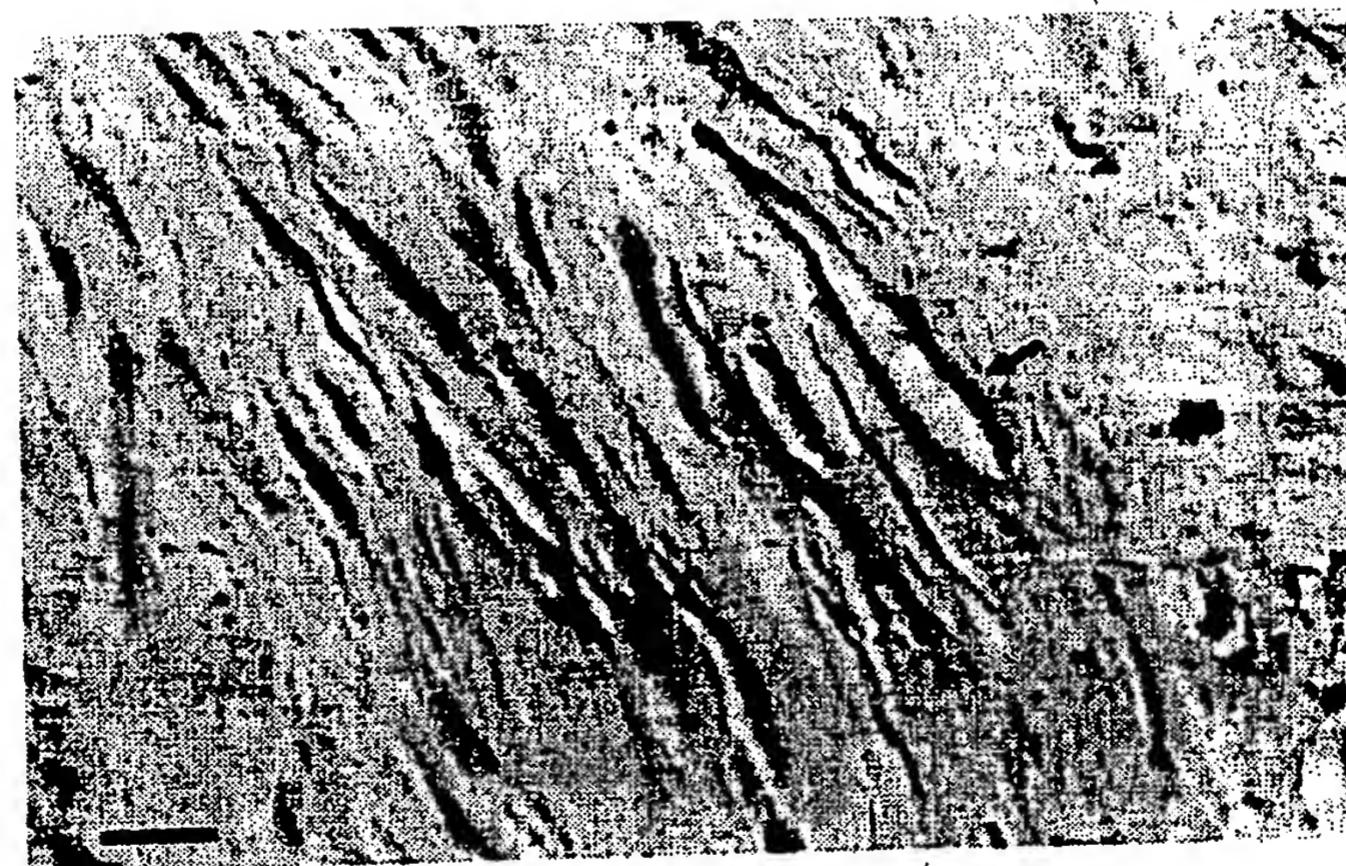
**FIG. 8**



**FIG. 9**

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**FIG. 10A****FIG. 10B**

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**FIG. 11**

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**WO 01/22978 A3**

(54) Title: AUTOLOGOUS MARROW STEM CELL (MSC) TRANSPLANTATION FOR MYOCARDIAL REGENERATION

(57) Abstract: The present invention relates to a method of improving cardiac function in a patient with heart failure without eliciting an immune response and without sacrificing the patient's skeletal muscle; which comprises the step of transplanting autologous bone marrow stroma cells (MSCs) into said patient's myocardium to grow new muscle fibers. The method may further comprise the step of using cell labeling technique to confirm survival and differentiation of implanted MSCs, and to identify said MSCs phenotype by both morphology and molecular markers. The method may further comprise examining the effects of the micro-environment of implanted MSCs on their differentiation and phenotype expression. The method may further comprise examining functional contribution of MSCs implanted into an ischemic segment of the myocardium.



